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(54) Title: NUCLEIC ACID SENSOR MOLECULES

(57) Abstract: Nucleic acid sensor molecules and methods are disclosed for the detection and amplification of signaling agents using enzymatic nucleic acid constructs, including hammerhead enzymatic nucleic acid molecules, inozymes, G-cleaver enzymatic nucleic acid molecules, zinzymes, amberzymes and DNAzymes; kits for detection and amplification; use in diagnostics, nucleic acid circuits, nucleic acid computers, and other uses are disclosed.

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## NUCLEIC ACID SENSOR MOLECULES

This invention claims priority from Usman *et al.*, USSN (60/187,128), filed March 6, 2000, entitled "A PROCESS FOR THE DETECTION OF NUCLEIC ACID USING NUCLEIC ACID CATALYSTS". This application is hereby incorporated by reference herein in its entirety including the drawings.

### Field of the Invention

This invention relates to novel molecular sensors that utilize enzymatic nucleic acid constructs whose activity can be modulated by the presence or absence of various signaling agents. The present invention further relates to the use of the enzymatic nucleic acid constructs as molecular sensors capable of modulating the activity, function, or physical properties of other molecules. The invention also relates to the use of the enzymatic nucleic acid constructs as a diagnostic application, useful in identifying signaling agents in a variety of applications, for example, in clinical, industrial, environmental, agricultural and/or research settings. The invention further relates to the use of the nucleic acid sensor constructs as a tool to identify the presence of genes and/or gene products which are indicative of a particular genotype and/or phenotype, for example a disease state, infection, or related condition within patients. In addition, the invention relates to the use of nucleic acid sensor molecules in nucleic acid-based electronics, including nucleic acid-based circuits and computers.

### Background of the Invention

The following is a brief description of diagnostic and sensor-based applications for nucleic acids. This summary is not meant to be complete but is provided only for understanding of the invention that follows. This summary is not an admission that all of the work described below is prior art to the claimed invention.

The detection of biomolecules, for example nucleic acids, can be highly beneficial in the diagnosis of diseases or medical disorders. By determining the presence of a specific nucleic acid sequence, investigators can confirm the presence of a virus, bacterium, genetic mutation, and other conditions that can relate to a disease. Assays for nucleic acid sequences can range from simple methods for detection, such as northern blot hybridization using a radiolabeled or fluorescent probe to detect the presence of a nucleic acid molecule, to the use of polymerase chain reaction (PCR) to amplify a small quantity of a specific nucleic acid to the point at which it can be used for detection of the sequence by hybridization techniques. The polymerase chain

reaction, uses DNA polymerases to logarithmically amplify the desired sequence (U.S. 4,683,195; U.S. 4,683,202) using prefabricated primers to locate specific sequences. Nucleotide probes can be labeled using dyes, fluorescent, chemiluminescent, radioactive, or enzymatic labels which are commercially available. These probes can be used to detect by hybridization, the expression of a gene or related sequences in cells or tissue samples in which the gene is a normal component, as well as to screen sera or tissue samples from humans suspected of having a disorder arising from infection with an organism, or to detect novel or altered genes as might be found in tumorigenic cells. Nucleic acid primers can also be prepared which, with reverse transcriptase or DNA polymerase and PCR, can be used for detection of nucleic acid molecules that are present in very small amounts in tissues or fluids.

PCR utilizes protein enzymes (DNA polymerase) to detect specific nucleotide sequences. PCR has several disadvantages, for example requiring a high degree of technical competence for reliability, high reagent costs, and sensitivity to contamination resulting in false positives.

Another class of enzymes which can be utilized for diagnostic and sensor purposes are enzymatic nucleic acid molecules (Kuwabara *et al.*, 2000, *Curr. Opin. Chem. Bio.*, 4, 669; Porta *et al.*, 1995, *Biochemistry*, 13, 161; Soukup *et al.*, 1999, *TIBTECH*, 17, 469; Marshall *et al.*, 1999, *Nature Struc Biol.*, 6, 992). The enzymatic nature of an enzymatic nucleic acid molecule can be advantageous over other sensor technologies, since the concentration of analyte necessary to generate a detectable response can be lower than that required with other sensor systems which can require amplification steps. This advantage reflects the ability of the enzymatic nucleic acid molecule to act enzymatically. Thus, a specific enzymatic nucleic acid molecule is able to amplify a given signal in response to a single recognition event. Such enzymatic nucleic acid-based sensor molecules are often referred to in the art as allosteric ribozymes or allosteric DNazymes.

In addition, the enzymatic nucleic acid molecule is a highly specific sensor molecule that can be engineered to respond to a variety of different signaling events. The use of *in vitro* selection techniques can be applied to the selection of new enzymatic nucleic acid molecules that are capable of allosteric modulation. Previous work in this area has focused on combining known aptamer and enzymatic nucleic acid molecule sequences (Breaker, International PCT Publication No. WO 98/2714). Later work has revealed bridge sequences that connect the receptor and enzymatic sequence domains together. These bridging sequences function such that binding of a ligand to the receptor domain triggers a conformational change within the bridge, thus modulating phosphodiester cleavage activity of the adjoining enzymatic sequence (Breaker, International PCT Publication No. WO 00/26226).

George *et al.*, US Patent Nos. 5,834,186 and 5,741,679, describe regulatable RNA molecules whose activity is altered in the presence of a ligand.

Shih *et al.*, US Patent No. 5,589,332, describe a method for the use of ribozymes to detect macromolecules such as proteins and nucleic acid.

Nathan *et al.*, US Patent No 5,871,914, describe a method for detecting the presence of an assayed nucleic acid based on a two component ribozyme system containing a detection ensemble and an RNA amplification ensemble.

Nathan and Ellington, International PCT publication No. WO 00/24931, describe the detection of an analyte by a catalytic nucleic acid sequence which converts a nucleic acid substrate to a catalytic nucleic acid product in the presence of the analyte. The catalytic nucleic acid product is then amplified, by PCR.

Sullenger *et al.*, International PCT publication No. WO 99/29842, describe nucleic acid mediated RNA tagging and RNA revision.

#### Summary of the Invention

The present invention relates to nucleic acid-based molecular sensors whose activity can be modulated by the presence or absence of various signaling agents, ligands, and/or target signaling molecules. The invention further relates to a method for the detection of specific target signaling molecules such as nucleic acid molecules, proteins, peptides, antibodies, polysaccharides, lipids, sugars, metals, microbial or cellular metabolites, analytes, pharmaceuticals, and other organic and inorganic molecules using nucleic acid sensor molecules in a variety of analytical settings, including clinical, industrial, veterinary, genomics, environmental, and agricultural applications. The invention further relates to the use of the nucleic acid sensor molecule as molecular sensors capable of modulating the activity, function, or physical properties of other molecules. The present invention also contemplates the use of the nucleic acid sensor molecule constructs as molecular switches, capable of inducing or negating a response in a system, for example in a nucleic acid-based circuit or computer.

The invention further relates to the use of nucleic acid sensor molecules in a diagnostic application to identify the presence of a target signaling molecule such as a gene and/or gene products which are indicative of a particular genotype and/or phenotype, for example, a disease state, infection, or related condition within patients or patient samples. The invention also relates to a method for the diagnosis of disease states or physiological abnormalities related to the expression of viral, bacterial or cellular RNA and DNA.



Diagnostic applications of the nucleic acid sensor molecules include the use of the nucleic acid sensor molecules for prospective diagnosis of disease, prognosis of therapeutic effect and/or dosing of a drug or class of drugs, prognosis and monitoring of disease outcome, monitoring of patient progress as a function of an approved drug or a drug under development, patient surveillance and screening for drug and/or drug treatment. Diagnostic applications include the use of nucleic acid sensors for research, development and commercialization of products for the rapid detection of macromolecules, such as mammalian viral nucleic acids, prions and viroids for the diagnosis of diseases associated with viruses, prions and viroids in humans and animals.

Nucleic acid sensor molecules can also be used in assays to assess the specificity, toxicity and effectiveness of various small molecules, nucleoside analogs, or non-nucleic acid drugs, or doses of a specific small molecules, nucleoside analogs or nucleic acid and non-nucleic acid drugs, against validated targets or biochemical pathways and include the use of nucleic acid sensors in assays involved in high-throughput screening, biochemical assays, including cellular assays, in vivo animal models, clinical trial management, and for mechanistic studies in human clinical studies. The nucleic acid sensor can also be used for the detection of pathogens, biochemicals, for example proteins, organic compounds, or inorganic compounds, in humans, plants, animals or samples therefrom, in connection with environmental testing or detection of biohazards. The use of the nucleic acid sensor molecules in other applications such a functional genomics, target validation and discovery, agriculture or diagnostics, for example the diagnosis of disease, or the prevention or treatment of human or animal disease is also contemplated.

In one embodiment, the invention features a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components, wherein, in response to an interaction of a target signaling molecule with the nucleic acid sensor molecule, the enzymatic nucleic acid component catalyzes a chemical reaction involving covalent attachment of at least a portion of a reporter molecule to the nucleic acid sensor molecule.

In another embodiment, the invention features a method, comprising the steps of: (a) contacting a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components, in which the enzymatic nucleic acid component catalyzes a chemical reaction in response to an interaction between a target signaling molecule and the nucleic acid sensor molecule, with a system under conditions suitable for the enzymatic nucleic acid component to catalyze a chemical reaction involving the attachment of at least a portion of a reporter molecule to the nucleic acid sensor molecule in the presence of a target signaling agent; and (b) assaying for the attachment of the reporter molecule to the nucleic acid sensor molecule.

In another embodiment, the invention features a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components, wherein, in response to an interaction of a target signaling molecule with the nucleic acid sensor molecule, the enzymatic nucleic acid component can carry out a chemical reaction involving isomerization of at least a portion of a reporter molecule.

In another embodiment, the invention features a method, comprising the steps of: (a) contacting a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components, wherein in response to an interaction of a target signaling molecule with the nucleic acid sensor molecule, the enzymatic nucleic acid component can carry out a chemical reaction involving isomerization of a reporter molecule, with a system under conditions suitable for the enzymatic nucleic acid component of the nucleic acid sensor molecule to isomerize of at least a portion of the reporter molecule in the presence of a target signaling agent; and (b) assaying for the isomerization reaction.

In another embodiment, the invention features a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components, wherein in response to an interaction of a target signaling molecule with the nucleic acid sensor molecule, the enzymatic component catalyses a chemical reaction on a non-oligonucleotide-based portion of a reporter molecule. For example, the chemical reaction catalyzed by the enzymatic compound can be a phosphorylation or dephosphorylation reaction.

In another embodiment, the invention features a method comprising the steps of: (a) contacting a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components, in which the enzymatic component catalyses a chemical reaction involving phosphorylation of a non-oligonucleotide-based portion of a reporter molecule in response to an interaction of a target signaling molecule with the nucleic acid sensor molecule, with a system under conditions suitable for the enzymatic nucleic acid component to phosphorylate a component of the reporter molecule in the presence of a target signaling agent; and (b) assaying for the phosphorylation reaction.

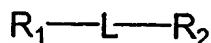
In another embodiment, the invention features a method comprising the steps of: (a) contacting a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components, in which the enzymatic component catalyses a chemical reaction involving dephosphorylation of a non-oligonucleotide-based portion of a reporter molecule in response to an interaction of a target signaling molecule with the nucleic acid sensor molecule, with a system under conditions suitable for the enzymatic nucleic acid component to

dephosphorylate a component of the reporter molecule in the presence of a target signaling agent; and (b) assaying for the dephosphorylation reaction.

In one embodiment, the nucleic acid sensor molecule of the instant invention features an enzymatic component and a sensor component that are distinct moieties.

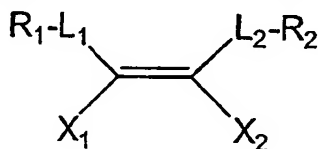
In another embodiment, the nucleic acid sensor molecule of the instant invention features a linker region that joins a sensor component to an enzymatic nucleic acid component.

In another embodiment, the invention features a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components, wherein, in response to an interaction of a target signaling molecule with the nucleic acid sensor molecule, the enzymatic nucleic acid component catalyzes a chemical reaction involving covalent attachment of at least a portion of a reporter molecule to at least a portion of the nucleic acid sensor molecule, wherein the reporter molecule comprises the formula:



wherein R1 is selected from the group consisting of alkyl, alkoxy, hydrogen, hydroxy, sulfhydryl, ester, anhydride, acid halide, amide, nitrile, phosphate, phosphonate, nucleoside, nucleotide, oligonucleotide; R2 is selected from the group consisting of molecular beacons, small molecules, fluorophores, chemophores, ionophores, radio-isotopes, photophores, peptides, proteins, enzymes, antibodies, nucleic acids, and enzymatic nucleic acids; L represents a linker which can be present or absent, and "-" represents a chemical bond.

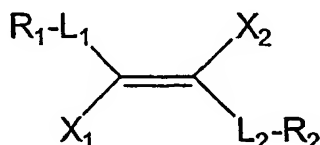
In another embodiment, the invention features a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components, wherein, in response to an interaction of a target signaling molecule with the nucleic acid sensor molecule, the enzymatic nucleic acid component can carry out a chemical reaction involving isomerization of at least a portion of a reporter molecule, wherein the reporter molecule comprises the formula:



wherein R1 and R2 each represent compounds, which can be the same or different, that generate a detectable signal or quench a detectable signal when an isomerization reaction is catalyzed, selected from the group consisting of molecular beacons, small molecules, fluorophores, chemophores, ionophores, radio-isotopes, photophores, peptides, proteins, enzymes, antibodies, nucleic acids, and enzymatic nucleic acids; L1 and L2 each represent a linker which can be the same or different and which can be present or absent; X1 and X2 each

represent an atom, compound, or molecule that can be the same or different, and “-” represents a chemical bond.

In another embodiment, the invention features a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components, wherein, in response to an interaction of a target signaling molecule with the nucleic acid sensor molecule, the enzymatic nucleic acid component can carry out a chemical reaction involving isomerization of at least a portion of a reporter molecule, wherein the reporter molecule comprises the formula:



wherein R1 and R2 each represent compounds, which can be the same or different, that generate a detectable signal or quench a detectable signal when an isomerization reaction is catalyzed, selected from the group consisting of molecular beacons, small molecules, fluorophores, chemophores, ionophores, radio-isotopes, photophores, peptides, proteins, enzymes, antibodies, nucleic acids, and enzymatic nucleic acids; L1 and L2 each represent a linker which can be the same or different and which can be present or absent; X1 and X2 represent an atom, compound, or molecule that can be the same or different, and “-” represents a chemical bond.

In one embodiment, the detection of a chemical reaction catalyzed by a nucleic acid sensor molecule of the instant invention is indicative of the presence of the target signaling molecule in a system.

In another embodiment, the absence of a chemical reaction catalyzed by a nucleic acid sensor molecule of the instant invention is indicative of a system lacking the target signaling molecule.

In another embodiment, the invention features a method comprising the steps of: (a) contacting a nucleic acid sensor molecule which comprises (i) an enzymatic nucleic acid component comprising a substrate binding region and a catalytic region; and (ii) a sensor component comprising a nucleic acid sequence that upon interacting with a complementary sequence in the enzymatic nucleic acid component, inhibits the activity of the enzymatic nucleic acid component, and a reporter molecule comprising a nucleic acid sequence complementary to the substrate binding region of the enzymatic nucleic acid component of the nucleic acid sensor molecule, with a system under conditions suitable for the enzymatic nucleic acid component of the nucleic acid sensor molecule to catalyze cleavage of the reporter molecule in the presence of a target signaling molecule; and (b) assaying for the cleavage reaction of step (a).

In another embodiment, the invention features a method comprising the steps of: (a) contacting a nucleic acid sensor molecule which comprises (i) an enzymatic nucleic acid component comprising a substrate binding region and a catalytic region; and (ii) a sensor component comprising a nucleic acid sequence that when interacts with a complementary sequence in the enzymatic nucleic acid component inhibits the activity of the enzymatic nucleic acid component and a reporter molecule comprising a nucleic acid sequence complementary to the substrate binding region of the enzymatic nucleic acid component of the nucleic acid sensor molecule, with a system under conditions suitable for the enzymatic nucleic acid component of the nucleic acid sensor molecule to catalyze a ligation reaction involving the reporter molecule in the presence of a target signaling molecule, and (b) assaying for the ligation reaction in step (a).

In one embodiment of the inventive method, the ligation reaction catalyzed by the nucleic acid sensor molecule causes at least a portion of a reporter molecule to be attached to the nucleic acid sensor molecule.

In another embodiment, the ligation reaction catalyzed by the nucleic acid sensor molecule causes at least a portion of a reporter molecule to be attached to a separate molecule. Suitable molecules include, for example, a separate nucleic acid molecule, peptide, protein, small molecule, biotin, or surface.

Also, in one embodiment of the inventive method, the cleavage of a reporter molecule catalyzed by the nucleic acid sensor molecule is indicative of the presence of the target signaling molecule in the system. In another embodiment, the absence of cleavage of a reporter molecule catalyzed by the nucleic acid sensor molecule is indicative of the system lacking the target signaling molecule.

In another embodiment of the inventive method, the ligation of a reporter molecule catalyzed by the nucleic acid sensor molecule is indicative of the presence of the target signaling molecule in the system. In another embodiment, the absence of ligation of a reporter molecule catalyzed by the nucleic acid sensor molecule is indicative of the system lacking the target signaling molecule.

In one embodiment, the system of the instant invention is an *in vitro* system. Preferably, the *in vitro* system is a sample derived from the group consisting of a patient, plant, water, beverage, food preparation, and soil.

In one embodiment, the target signaling molecule of the instant invention is an RNA, DNA, analog of RNA or analog of DNA. Preferably, the target signaling molecule of the instant invention is an RNA derived from a bacteria, virus, fungi, plant or mammalian genome.

In one embodiment, the enzymatic nucleic acid component of the nucleic acid sensor molecule is selected from the group consisting of hammerhead, hairpin, inozyme, G-cleaver, Zinzyme, RNase P EGS nucleic acid and Amberzyme motif. In another embodiment, the enzymatic nucleic acid component of the nucleic acid sensor molecule is a DNAzyme.

In one embodiment, the reporter molecule of the instant invention comprises a detectable label selected from the group consisting of chromogenic substrate, fluorescent labels, chemiluminescent labels, and radioactive labels and enzymes. Suitable enzymes include, for example, luciferase, horseradish peroxidase, and alkaline phosphatase.

In another embodiment, the reporter molecule of the instant invention is immobilized on a solid support. Suitable solid supports include silicon-based chips, silicon-based beads, controlled pore glass, polystyrene, cross-linked polystyrene, nitrocellulose, biotin, plastics, metals and polyethylene films.

In one embodiment the sensor component of the nucleic acid sensor molecule is RNA, DNA, analog of RNA or analog of DNA.

In another embodiment, the sensor component of the nucleic acid sensor molecule is covalently linked to the nucleic acid sensor molecule by a linker. Suitable linkers include one or more nucleotides, abasic moieties, polyethers, polyamines, polyamides, peptides, carbohydrates, lipids, and polyhydrocarbon compounds, and any combination thereof.

In another embodiment, the sensor component of the nucleic acid sensor molecule is not covalently linked to the nucleic acid sensor molecule.

In another embodiment, the reporter molecule of the instant invention is RNA, DNA, RNA analog, or DNA analog.

In another embodiment, the invention features a kit comprising: (a) a nucleic acid sensor molecule which comprises (i) an enzymatic nucleic acid component comprising a substrate binding region and a catalytic region; and (ii) a sensor component comprising a nucleic acid which interacts with a complementary sequence in the enzymatic nucleic acid component to inhibit the activity of the enzymatic nucleic acid component; and (b) a reporter molecule that can be modified, i.e., cleaved, ligated, polymerized, isomerized, phosphorylated, and/or dephosphorylated by the enzymatic nucleic acid component of the nucleic acid sensor molecule in the presence of a target signaling molecule, wherein the reporter molecule comprises a chemical moiety capable of emitting a detectable signal upon its modification.

In another embodiment, the invention features a kit which comprises: (a) a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components; and (b) a reporter molecule, wherein, in response to an interaction of a target

signaling molecule with the nucleic acid sensor molecule, the enzymatic nucleic acid component catalyzes a chemical reaction involving covalent attachment of at least a portion of a reporter molecule to the nucleic acid sensor molecule.

In another embodiment, the invention features a kit which comprises: (a) a nucleic acid sensor molecule comprising, an enzymatic nucleic acid component and one or more sensor components; and (b) a reporter molecule, wherein in response to an interaction of a target signaling molecule with the nucleic acid sensor molecule, the enzymatic nucleic acid component is capable of carrying out a chemical reaction involving isomerization of at least a portion of a reporter molecule.

In another embodiment, the invention features a kit which comprises: (a) a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components; and (b) a reporter molecule having a non-oligonucleotide-based portion, wherein, in response to an interaction of a target signaling molecule with the nucleic acid sensor molecule, the enzymatic component catalyses a chemical reaction involving phosphorylation of a non-oligonucleotide-based portion of a reporter molecule.

In another embodiment, the invention features a kit which comprises: (a) a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components; and (b) a reporter molecule having a non-oligonucleotide-based portion, wherein, in response to an interaction of a target signaling molecule with the nucleic acid sensor molecule, the enzymatic component catalyses a chemical reaction involving dephosphorylation of a non-oligonucleotide-based portion of a reporter molecule.

In another embodiment, the invention features a method comprising the step of contacting one or more components of a kit of the instant invention with a system under conditions suitable for the reporter molecule in the kit to be cleaved by the nucleic acid sensor molecule in the kit in the presence of a target signaling molecule.

In another embodiment, the invention features a method comprising the step of contacting one or more components of a kit of the instant invention with a system under conditions suitable for at least a portion of the reporter molecule in the kit to be covalently attached to the nucleic acid sensor molecule in the kit in the presence of a target signaling molecule.

In another embodiment, the invention features a method comprising the step of contacting one or more components of a kit of the instant invention with a system under conditions suitable for at least a portion of the reporter molecule in the kit to be isomerized by the nucleic acid sensor molecule in the kit in the presence of a target signaling molecule.

In another embodiment, the invention features a method comprising the step of contacting one or more components of a kit of the instant invention with a system under conditions suitable for at least a portion of the reporter molecule in the kit to be phosphorylated by the nucleic acid sensor molecule in the kit in the presence of a target signaling molecule.

In another embodiment, the invention features a method comprising the step of contacting one or more components of a kit of the instant invention with a system under conditions suitable for at least a portion of the reporter molecule in the kit to be dephosphorylated by the nucleic acid sensor molecule in the kit in the presence of a target signaling molecule.

In another embodiment, the invention features a nucleic acid circuit including a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components, wherein, in response to an interaction of a target signaling agent with the nucleic acid sensor molecule, the enzymatic nucleic acid component catalyzes a chemical reaction involving ligation of at least a portion of a nucleic acid based component of the nucleic acid circuit.

In another embodiment, the invention features a nucleic acid circuit including a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components, wherein, in response to an interaction of a target signaling agent with the nucleic acid sensor molecule, the enzymatic nucleic acid component catalyzes a chemical reaction involving cleavage of at least a portion of a nucleic acid based component of the nucleic acid circuit.

In another embodiment, the invention features a nucleic acid computer comprising one or more nucleic acid circuits including a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components, wherein, in response to an interaction of a target signaling agent with the nucleic acid sensor molecule, the enzymatic nucleic acid component catalyzes a chemical reaction involving ligation of at least a portion of a nucleic acid based component of the nucleic acid circuit.

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In one embodiment, the signaling agent of the instant invention is current. In another embodiment, the signaling agent of the instant invention is voltage. In yet another embodiment, the signaling agent of the instant invention is impedance.

In one embodiment, the nucleic acid computer of the instant invention comprises a plurality of nucleic acid circuits that are arranged in a parallel array.

In one embodiment, the nucleic acid computer of the instant invention is used to detect a signaling agent. In another embodiment, the nucleic acid computer of the instant invention is used to solve a problem.

In one embodiment, the invention features a method comprising the steps of: (a) contacting a nucleic acid circuit of the invention with a signaling agent under conditions suitable for the nucleic acid sensor molecule to ligate at least a portion of a nucleic acid based component of the nucleic acid circuit; and (b) assaying for the ligation of step (a).

In another embodiment, the invention features a method comprising the steps of: (a) contacting the nucleic acid circuit of the invention with a signaling agent under conditions suitable for the nucleic acid sensor molecule to cleave at least a portion of a nucleic acid based component of the nucleic acid circuit; and (b) assaying for the cleavage of step (a).

In one embodiment, the ligation of a nucleic acid circuit by a nucleic acid sensor molecule of the invention is assayed by measuring parameters selected from the group consisting of current, voltage, capacitance, and impedance.

Also, in one embodiment, the cleavage of a nucleic acid circuit by a nucleic acid sensor molecule of the invention is assayed by measuring parameters selected from the group consisting of current, voltage, capacitance, and impedance.

In one embodiment, the invention features a method for isolating a nucleic acid sensor molecule of the instant invention, comprising the steps of: (a) contacting a random pool of nucleic acids with a target signaling molecule and a reporter molecule, and (b) isolating a nucleic acid sensor molecule that can catalyze a chemical reaction involving covalent attachment of at least a portion of the reporter molecule to the nucleic acid sensor molecule in the presence of the target signaling molecule.

In another embodiment, the invention features a method for isolating a nucleic acid sensor molecule of the instant invention comprising the steps of: (a) contacting a random pool of nucleic acids with a target signaling molecule and a reporter molecule, and (b) isolating a nucleic acid sensor molecule that can catalyze a chemical reaction involving ligation of at least a portion of the reporter molecule to the nucleic acid sensor molecule in the presence of the target signaling molecule.

In another embodiment, the invention features a method for isolating a nucleic acid sensor molecule of the instant invention comprising the steps of: (a) contacting a random pool of nucleic acids with a target signaling molecule and a non-oligonucleotide-based reporter molecule, and (b) isolating a nucleic acid sensor molecule that can catalyze a chemical reaction involving phosphorylation a non-oligonucleotide-based portion of the reporter molecule by the nucleic acid sensor molecule in the presence of the target signaling molecule.

In another embodiment, the invention features a method for isolating a nucleic acid sensor molecule of the instant invention, comprising the steps of: (a) contacting a random pool of nucleic acids with a target signaling molecule and a non-oligonucleotide-based reporter molecule, and (b) isolating a nucleic acid sensor molecule that can catalyze a chemical reaction involving dephosphorylation of a non-oligonucleotide-based portion of the reporter molecule by the nucleic acid sensor molecule in the presence of the target signaling molecule.

In one embodiment, the nucleic acid sensor molecule of the instant invention is attached to a surface. Preferably, the surface of the instant invention is selected from the group consisting of silicon-based chips, silicon-based beads, controlled pore glass, polystyrene, and cross-linked polystyrene.

In another embodiment, the method of the instant invention is carried out more than once.

#### Detailed Description of the Invention

The present invention features compositions and methods for the detection and/or amplification of specific target signaling agents and target signaling molecules in a system using nucleic acid sensor molecules.

In one embodiment, the present invention features a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components that, in response to an interaction of a target signaling agent, the enzymatic nucleic acid component catalyzes a chemical reaction in which the activity or physical properties of a reporter molecule is modulated. Preferably, the chemical reaction in which the activity or physical properties of a reporter molecule is modulated results in a detectable response.

In a preferred embodiment, the present invention features a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components, wherein, in response to an interaction of a target signaling agent or target signaling molecule with the nucleic acid sensor molecule, the enzymatic nucleic acid component catalyses a chemical reaction involving covalent attachment of at least a portion of a reporter molecule.

Preferably, the chemical reaction in which a reporter molecule is covalently attached to the nucleic acid sensor molecule is selected from the group consisting of ligation, transesterification,

phosphorylation, carbon-carbon bond formation, amide bond formation, peptide bond formation, and disulfide bond formation.

In another embodiment, the present invention features a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components, wherein, in response to an interaction of a target signaling molecule with the nucleic acid sensor molecule, the enzymatic nucleic acid component can carry out a chemical reaction that modulates the activity or properties of the reporter molecule. Preferably, the chemical reaction in which the activity of a reporter molecule is modulated is selected from the group consisting of phosphorylation, dephosphorylation, isomerization, polymerization, amplification, helicase activity, transesterification, ligation, hydration, hydrolysis, alkylation, dealkylation, halogenation, dehalogenation, esterification, deesterification, hydrogenation, dehydrogenation, saponification, desaponification, amination, deamination, acylation, deacylation, glycosylation, deglycosylation, silylation, desilylation, hydroboration, epoxidation, peroxidation, carboxylation, decarboxylation, substitution, elimination, oxidation, and reduction or a combination thereof.

In a preferred embodiment, the invention features a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components, wherein, in response to an interaction of a target signaling molecule with the nucleic acid sensor molecule, the enzymatic nucleic acid component can carry out a chemical reaction involving isomerization of at least a portion of a reporter molecule.

In another preferred embodiment, the invention features a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components, wherein, in response to an interaction of a target signaling molecule with the nucleic acid sensor molecule, the enzymatic component catalyses a chemical reaction on a non-oligonucleotide-based portion of a reporter molecule selected from the group consisting of phosphorylation and dephosphorylation.

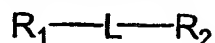
Preferably, the reporter molecule of the instant invention is selected from the group consisting of molecular beacons, small molecules, fluorophores, chemophores, ionophores, radio-isotopes, photophores, peptides, proteins, enzymes, antibodies, nucleic acids, and enzymatic nucleic acids or a combination thereof (see for example in Singh *et al.*, 2000, *Biotech.*, 29, 344; Lizardi *et al.*, US Patent Nos. 5,652,107 and 5,118,801).

Using such reporter molecules and others known in the art, the detectable response of the instant invention can be monitored by, for example, a change in fluorescence, color change, UV absorbance, phosphorescence, pH, optical rotation, isomerization, polymerization, temperature, mass, capacitance, resistance, and emission of radiation.

Detection of the target signaling event via the chemical reaction or the change in activity or physical properties of the reporter molecule can be assayed by methods known in the art. Amplification of the target signaling event via the chemical reaction or the change in activity or physical properties of the reporter molecule is accomplished by methods known in the art, for example, modulating polymerase activity. Modulation of polymerase activity can increase polymerization in a chemical reaction, for example, a polymerase chain reaction (PCR) system, resulting in amplification of a target signaling molecule or reporter molecule.

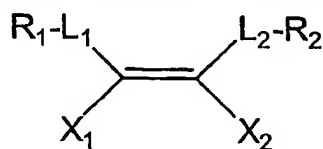
In one embodiment, a linker region (see, for example L in Figures 17 a,b,c) can join the nucleic acid sensor molecule to a reporter molecule, for example, via ligation activity of an enzymatic nucleic acid component of the nucleic acid sensor molecule in response to a target signaling agent's interaction with a sensor component of the nucleic acid sensor molecule.

In one preferred embodiment, the invention features a nucleic acid sensor molecule, wherein said reporter molecule comprises the formula:

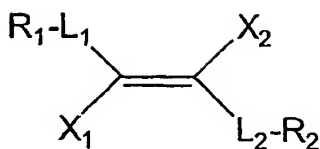


wherein R1 is selected from the group consisting of alkyl, alkoxy, hydrogen, hydroxy, sulfhydryl, ester, anhydride, acid halide, amide, nitrile, phosphate, phosphonate, nucleoside, nucleotide, oligonucleotide; R2 is selected from the group consisting of molecular beacons, small molecules, fluorophores, chemophores, ionophores, radio-isotopes, photophores, peptides, proteins, enzymes, antibodies, nucleic acids, and enzymatic nucleic acids; L represents a linker which can be present or absent, and "-" represents a chemical bond

In another preferred embodiment, the invention features a nucleic acid sensor molecule of claim 3, wherein said reporter molecule comprises the formula:



wherein R1 and R2 each represent compounds, which can be the same or different, that generate a detectable signal or quench a detectable signal when an isomerization reaction is catalyzed, selected from the group consisting of molecular beacons, small molecules, fluorophores, chemophores, ionophores, radio-isotopes, photophores, peptides, proteins, enzymes, antibodies, nucleic acids, and enzymatic nucleic acids; L1 and L2 each represent a linker which can be the same or different and which can be present or absent; X1 and X2 each represent an atom, compound, or molecule that can be the same or different, and "-" represents a chemical bond. In another preferred embodiment, the invention features a nucleic acid sensor molecule of claim 3, wherein said reporter molecule comprises the formula:



wherein R1 and R2 each represent compounds, which can be the same or different, that generate a detectable signal or quench a detectable signal when an isomerization reaction is catalyzed, selected from the group consisting of molecular beacons, small molecules, fluorophores, chemophores, ionophores, radio-isotopes, photophores, peptides, proteins, enzymes, antibodies, nucleic acids, and enzymatic nucleic acids; L1 and L2 each represent a linker which can be the same or different and which can be present or absent; X1 and X2 represent an atom, compound, or molecule that can be the same or different, and “-” represents a chemical bond.

Preferably, the reaction catalyzed by the enzymatic nucleic acid component of the nucleic acid sensor or nucleic acid sensor molecule with the reporter molecule of the invention features catalytic activity, for example, cleavage activity, ligation activity, isomerization activity, phosphorylation activity, dephosphorylation activity, amplification activity, and/or polymerase activity.

The invention also features a method comprising the steps of: (a) contacting a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components, and a reporter molecule with a system under conditions suitable for the enzymatic nucleic acid component of the nucleic acid sensor molecule to attach at least a portion of the reporter molecule to the nucleic acid sensor molecule in the presence of a target signaling agent; and (b) assaying for the attachment of the reporter molecule to the nucleic acid sensor molecule.

In another embodiment, the invention features a method comprising the steps of: (a) contacting a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components, and a reporter molecule with a system under conditions suitable for the enzymatic nucleic acid component of the nucleic acid sensor molecule to isomerize at least a portion of the reporter molecule in the presence of a target signaling agent; and (b) assaying for the isomerization reaction.

In yet another embodiment, the invention features a method comprising the steps of: (a) contacting a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components, and a reporter molecule with a system under conditions suitable for the enzymatic nucleic acid component of the nucleic acid sensor molecule to phosphorylate a non-oligonucleotide-based portion of the reporter molecule in the presence of a target signaling agent; and (b) assaying for the phosphorylation reaction.

In still another embodiment, the invention features a method comprising the steps of: (a) contacting a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components, and a reporter molecule with a system under conditions suitable for the enzymatic nucleic acid component of the nucleic acid sensor molecule to dephosphorylate a non-oligonucleotide-based portion of the reporter molecule in the presence of a target signaling agent; and (b) assaying for the dephosphorylation reaction.

In any of the above-described inventive methods, the system can be an *in vitro* system. Preferably, the *in vitro* system is a sample derived from the group consisting of a patient, plant, water, beverage, food preparation, and soil. In any of the above-described inventive methods, the enzymatic nucleic acid component of said nucleic acid sensor molecule can be a hammerhead, hairpin, inozyme, G-cleaver, Zinzyme, RNase P EGS nucleic acid and Amberzyme motif. Also, in any of the above-described inventive methods, the enzymatic nucleic acid component of said nucleic acid sensor molecule can be a DNAzyme.

In any of the above-described methods, the detection of a chemical reaction can be indicative of the presence of the target signaling molecule in the system. In any of the above-described methods, the absence of a chemical reaction is indicative of the system lacking the target signaling molecule.

Preferably, the reporter molecule of the instant invention is selected from the group consisting of molecular beacons, small molecules, fluorophores, chemophores, ionophores, radio-isotopes, photophores, peptides, proteins, enzymes, antibodies, nucleic acids, and enzymatic nucleic acids or a combination thereof (see for example in Singh *et al.*, 2000, *Biotech.*, 29, 344; Lizardi *et al.*, US Patent Nos. 5,652,107 and 5,118,801).

Using such reporter molecules and others known in the art, the detectable response of the instant invention can be monitored by, for example, a change in fluorescence, color change, UV absorbance, phosphorescence, pH, optical rotation, isomerization, polymerization, temperature, mass, capacitance, resistance, and emission of radiation.

Detection of the target signaling event via the chemical reaction or the change in activity or physical properties of the reporter molecule can be assayed by methods known in the art. Amplification of the target signaling event via the chemical reaction or the change in activity or physical properties of the reporter molecule is accomplished by methods known in the art, for example, modulating polymerase activity. Modulation of polymerase activity can increase polymerization in a chemical reaction, for example, a polymerase chain reaction (PCR) system, resulting in amplification of a target signaling molecule or reporter molecule.

The present invention features a nucleic acid-based sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components. The nucleic acid sensor molecule is selected for having catalytic activity only through interaction with a target signaling agent such that in response to an interaction of the target signaling agent with at least one sensor component, the enzymatic portion of the nucleic acid sensor molecule catalyzes a chemical reaction.

In one embodiment, the nucleic acid sensor molecule comprises an enzymatic nucleic acid component and one or more sensor components, wherein the enzymatic nucleic acid component and sensor component(s) are distinct moieties.

In one embodiment, the nucleic acid sensor molecule comprises an enzymatic nucleic acid component and one or more sensor components, wherein the distinct enzymatic nucleic acid component and sensor component(s) are joined by a linker region. Thus, in one embodiment, a linker region joins one or more enzymatic nucleic acid components to one or more sensor components in the nucleic acid sensor molecules of the instant invention.

As discussed above, the chemical reaction carried out by the nucleic acid sensor molecule can comprise a reaction in which a reporter molecule or a portion of a reporter molecule becomes covalently attached to the nucleic acid sensor molecule. Thus, in another embodiment, the nucleic acid sensor molecule comprises an enzymatic nucleic acid component and one or more sensor components, wherein the distinct enzymatic nucleic acid component and sensor component(s) are joined by a covalent bond. In one embodiment, the chemical reaction carried out by the nucleic acid sensor molecule comprises a reaction in which a reporter molecule becomes covalently attached to the nucleic acid sensor molecule that is immobilized on a solid support or surface. Suitable solid surfaces include silicon-based chips, silicon-based beads, controlled pore glass, polystyrene, and cross-linked polystyrene nitrocellulose, biotin, plastics, metals and polyethylene films. In another embodiment, the nucleic acid sensor molecule comprises an enzymatic nucleic acid component and one or more sensor components, wherein a sensor component of a nucleic acid sensor molecule of the instant invention is an integral part of the enzymatic nucleic acid component of the nucleic acid sensor molecule. Specifically, in preferred embodiments, one or more sensor components of a nucleic acid sensor molecule shares sequence with the enzymatic nucleic acid component of the nucleic acid sensor molecule and is necessary for the activity of the enzymatic nucleic acid component. The sensor component can also be part of the enzymatic nucleic acid component of the nucleic acid sensor molecule.

In the presence of a target signaling molecule, the sensor component activates or facilitates a chemical reaction. Alternatively, in the presence of a target signaling molecule, the sensor component inhibits a chemical reaction from taking place.

In preferred embodiments, the invention features the use of at least one reporter molecule, at least one target signaling molecule, and a nucleic acid sensor molecule which is comprised of an enzymatic nucleic acid component joined by a linker to one or more sensor components, where a sensor component, for example, is complementary to one or more sequences within the enzymatic nucleic acid component. The enzymatic nucleic acid component's ability, in the nucleic acid sensor or nucleic acid sensor molecule, to catalyze a reaction is inhibited by the interaction of one or more sensor components. However, in the presence of one or more distinct target signaling molecules, the sensor component interacts with its respective target signaling molecule preferentially, allowing the nucleic acid sensor molecule to interact with a reporter molecule to catalyze a reaction. A catalytic reaction then takes place on the reporter molecule, for example, cleavage or ligation of the reporter molecule, the rate of which can then be measured by standard assays described herein and otherwise well known in the art.

In another preferred embodiment, the invention features a method for the detection and/or amplification of specific target signaling molecules in a system using at least one reporter molecule, at least one target signaling molecule, and a nucleic acid sensor molecule which comprises an enzymatic nucleic acid component and at least one separate sensor component, where the sensor component or components interacts with one or more sequences within the nucleic acid sensor molecule. The enzymatic nucleic acid component's ability, in the nucleic acid sensor molecule, to catalyze a reaction is inhibited by the interaction of at least one sensor component. However, in the presence of a target signaling molecule, the sensor component preferentially interacts with the enzymatic nucleic acid component, which allows the nucleic acid sensor molecule to interact with a reporter molecule and become functional. A catalytic reaction then takes place on the reporter molecule, for example, cleavage or ligation of the reporter molecule, the rate of which can then be measured by standard assays described herein and otherwise well known in the art.

In a preferred embodiment, the invention features a method for the detection and/or amplification of a specific target signaling molecule in a system using at least one reporter molecule, at least one target signaling molecule, and a nucleic acid sensor molecule which comprises an enzymatic nucleic acid component. The nucleic acid sensor molecule is selected for having catalytic activity only through interaction with the target signaling molecule. In the absence of the target signaling molecule, the nucleic acid sensor molecule is inactive. In the



presence of a target signaling molecule the nucleic acid sensor molecule can adopt an active conformation and become functional. A catalytic reaction then takes place on the reporter molecule, for example, cleavage or ligation of the reporter molecule, the rate of which can then be measured by standard assays well known in the art. Alternatively, the nucleic acid sensor molecule can be selected to be inhibited through interaction with the target signaling molecule, such that interaction with the target causes the nucleic acid sensor molecule to adopt an inactive conformation and become non-active.

Thus in one embodiment, the present invention features a method comprising the steps of:

(a) contacting a nucleic acid sensor molecule which comprises: (i) an enzymatic nucleic acid component comprising a substrate binding region and a catalytic region; and (ii) a sensor component comprising a nucleic acid sequence that upon interacting with a complementary sequence in the enzymatic nucleic acid component inhibits the activity of the enzymatic nucleic acid component, and a reporter molecule comprising a nucleic acid sequence complementary to the substrate binding region of the enzymatic nucleic acid component of the nucleic acid sensor molecule with a system under conditions suitable for the enzymatic nucleic acid component of the nucleic acid sensor molecule to catalyze cleavage of the reporter molecule in the presence of a target signaling molecule; and (b) assaying for the cleavage reaction of step (a).

In one embodiment of the inventive method, the cleavage of the reporter molecule is indicative of the presence of the target signaling molecule in the system. The absence of cleavage of the reporter molecule is indicative of the system lacking the target signaling molecule.

In another embodiment, the present invention features a method comprising the steps of:

(a) contacting a nucleic acid sensor molecule which comprises: (i) an enzymatic nucleic acid component comprising a substrate binding region and a catalytic region; and (ii) a sensor component comprising a nucleic acid sequence that upon interacting with a complementary sequence in the enzymatic nucleic acid component inhibits the activity of the enzymatic nucleic acid component, and a reporter molecule comprising a nucleic acid sequence complementary to the substrate binding region of the enzymatic nucleic acid component of the nucleic acid sensor molecule with a system under conditions suitable for the enzymatic nucleic acid component of the nucleic acid sensor molecule to catalyze a ligation reaction involving the reporter molecule in the presence of a target signaling molecule; and (b) assaying for the ligation reaction in step (a).

In one embodiment of the inventive method, the ligation reaction causes at least a portion of the reporter molecule to be attached to the nucleic acid sensor molecule. In another embodiment, the ligation reaction causes at least a portion of the reporter molecule to be attached to a separate molecule. Also, in one embodiment of the inventive method, the ligation of the

reporter molecule is indicative of the presence of the target signaling molecule in the system. The absence of ligation of the reporter molecule is indicative of the system lacking the target signaling molecule.

In any of the above-described inventive methods, the system can be an *in vitro* system. Preferably, the *in vitro* system is a sample derived from the group consisting of a patient, plant, water, beverage, food preparation, and soil.

In any of the above described methods, the target signaling molecule can be an RNA, DNA, analog of RNA or analog of DNA. Thus, for example, the reporter molecule can be an RNA, DNA, RNA analog, or DNA analog. Also, in any of the described methods, wherein the targeting signaling molecule is an RNA, preferably the RNA is derived from a bacteria, virus, fungi, plant or mammalian genome. In one embodiment, the invention features a method of detecting and/or amplifying target signaling molecules, wherein said target signaling molecule is a nucleic acid sequence such as RNA and/or DNA, in a system, preferably a mammalian system, comprising the steps of (1) contacting the system with the nucleic acid sensor molecule and the reporter molecule under conditions suitable for the target signaling molecule, if present in the sample, to interact with the sensor component of the nucleic acid sensor molecule, such that the enzymatic nucleic acid component of the sensor molecule can interact with the reporter molecule to catalyze a reaction; and (2) measuring of the extent of the reaction catalyzed by the enzymatic nucleic acid component of the sensor molecule, indicating the presence of the target signaling molecule. If the target signaling molecule is not present in the sample, then no reaction above the background will be detected. The reporter molecule can be contacted with the system after the system is allowed to interact with the nucleic acid sensor molecule.

In another embodiment, the invention features a method of detecting and/or amplifying a target signaling molecule, wherein the target signaling molecule is RNA sequence derived from a virus, bacteria, fungi, mycoplasma or other infectious disease agent, in a system, where the system is a biological sample from a patient, animal, blood, food material, water, and/or other potential sources for infectious disease agents. The method comprises the steps of (1) contacting the system with the nucleic acid sensor molecule, where the nucleic acid sensor molecule comprises an sensor component and an enzymatic nucleic acid component, under conditions suitable for preferential interaction of the sensor component with the target signaling molecule that can be present in the system; (2) contacting the system with a reporter molecule under conditions suitable for the enzymatic nucleic acid component of the nucleic acid sensor molecule to catalyze a reaction with the reporter molecule; and (3) detecting the target signaling molecule by measuring any reaction catalyzed in step (2).

In another embodiment, the invention features a method of the detecting and/or amplifying a target signaling molecule, wherein the target signaling molecule is RNA sequence derived from a virus, bacteria, fungi, mycoplasma or other infectious disease agent, in a system, where the system is a biological sample from a patient, animal, blood, food material, water, and/or other potential sources for infectious disease agents. The method comprises the steps of (1) contacting the reporter molecule with a mixture, comprising the system and the nucleic acid sensor molecule, under conditions suitable for the active configuration of the enzymatic nucleic acid component of the nucleic acid sensor molecule to interact with the reporter molecule to catalyze a reaction; and (2) detecting the target signaling molecule by measuring the reaction catalyzed in step (1). If the target signaling molecule is not present in the system, then the enzymatic nucleic acid component will not catalyze a reaction with the reporter molecule and there will not be a signal to measure.

In another embodiment, one or more nucleic acid sensor molecules are attached to a solid support, for example, a silicon-based surface. Each nucleic acid sensor molecule can be attached via one of its termini by a spacer molecule to allow the nucleic acid sensor molecule to adopt the appropriate conformations without hindrance from the underlying solid support. A test mixture is contacted with one or more nucleic acid sensor molecules, and the mixture is contacted with the solid support. Measurement of a signal generated by the nucleic acid sensor molecule in response to interaction with a target signaling molecule at each address of the array reveals the concentration of each target signaling molecule in the test mixture.

In any of the above methods, the enzymatic nucleic acid component of said nucleic acid sensor molecule can be a hammerhead, hairpin, inozyme, G-cleaver, Zinzyme, RNase P EGS nucleic acid and Amberzyme motif.

In any of the above methods, the enzymatic nucleic acid component of said nucleic acid sensor molecule can be a DNAzyme.

In any of the above methods, the reporter molecule can comprise a detectable label selected from the group consisting of chromogenic substrate, fluorescent labels, chemiluminescent labels, and radioactive labels.

In any of the above methods, the reporter molecule can be immobilized on a solid support, preferably comprising silicon-based chips, silicon-based beads, controlled pore glass, polystyrene, cross-linked polystyrene, nitrocellulose, biotin, plastics, metals and polyethylene films.

In one embodiment of the inventive method, the sensor component of the nucleic acid sensor molecule is RNA, DNA, analog of RNA or analog of DNA.

In another embodiment, the sensor component of the nucleic acid sensor molecule is covalently linked to the nucleic acid sensor molecule by a linker. Suitable linkers include, for example, one or more nucleotides, abasic moiety, polyether, polyamine, polyamide, peptide, carbohydrate, lipid, and polyhydrocarbon compounds, and any combination thereof.

In another embodiment, the sensor component of the nucleic acid sensor molecule is not covalently linked to the nucleic acid sensor molecule.

In one embodiment, the nucleic acid sensor molecules of the invention are used to detect target signaling agents involved in human and animal disease, for example viruses, bacteria, proteins, other pathogens and toxins. Examples of viral target signaling agents include but are not limited to Hepatitis C virus (HCV), Hepatitis B virus (HBV), human immunodeficiency virus (HIV), human papilloma virus (HPV), poliovirus, West Nile virus (WNV), cytomegalovirus (CMV), Herpes Simplex Virus (HSV), respiratory syncytial virus (RSV), influenza virus, rhinovirus, foot and mouth disease virus, ebola virus, dengue fever virus, feline leukemia virus (FLV), and others. Examples of bacterial target signaling agents include but are not limited to Corynebacteria, Pneumococci, Streptococci, Staphylococci, enteric bacilli, mycobacteria, spirochetes, chlamydiae, and others. Examples of protein target signaling agents include but are not limited to prions, for example CVJ and BSE associated prions, signal transduction proteins, tyrosine kinases, phosphatases, phosphorylases, dephosphorylases, polymerases and others. Examples of other parasite target signaling agents include but are not limited to pathogenic agents related to malaria, lyme disease (*Borrelia burgdorferi*), sleeping sickness, giardia, and cryptosporidia. Examples of toxin target signaling agents include but are not limited to lead, mercury, asbestos, pesticides, herbicides, PCBs, and other organic and inorganic compounds.

The present invention also provides kits for the detection of particular targets in test mixtures. The kit comprises separate components containing solutions of a nucleic acid sensor molecule specific for a particular target signaling agent, and containing solutions of the appropriate reporter molecules. In some embodiments, the kit comprises a solid support to which is attached the nucleic acid sensor molecule to the particular target. In further embodiments, the kit further comprises a component containing a standardized solution of the target. With this solution, it is possible for the user of the kit to prepare a graph or table of the detectable signal (for example, fluorescence units vs. target concentration); this table or graph is then used to determine the concentration of the target in the test mixture. Devices that automate the manipulation of such kits, perform the repeated function of the kits, combine various steps of kits, or that generate data from the kits are further contemplated by the instant invention.

In one embodiment, the invention features the use of nucleic acid sensor molecules in nucleic acid based electronics, including nucleic acid-based switches, semiconductor circuits, and computers. The present invention also provides for the detection of signaling agents by means of nucleic acid circuit arrays, including the use of nucleic acid sensor molecules in nucleic acid based switches, semiconductor circuits, and computers. Recent research has indicated the capacity for nucleic acids, specifically DNA, to act as a conductor, semiconductor, or insulator based on nucleotide sequence (Porath *et al.*, 2000, *Nature*, 403, 635-638; Kelley and Barton, 1999, *Science*, 283, 375-381; and Jortner *et al.*, 1998, *PNAS USA.*, 95, 12759-12765; and Geise *et al.*, 1999, *Angew. Chem, Int. Ed. Engl.*, 38, 996). The use of nucleic acid sensor molecules *in cis* or *in trans* in nucleic acid circuits is further contemplated by the instant invention. Such use can provide a means for creating complex nucleic acid based circuits, enabling the development of nano-computing devices, biosensors, and biologically integrated circuits for *in vitro* and *in vivo* use through the ability for the nucleic acid sensor molecule to either cleave or ligate a nucleic acid *in cis* or *in trans* in response to signaling agents.

In another embodiment, the nucleic acid sensor molecules are used in DNA computing applications. For example, the use of unique sequences of nucleic acid can be used to solve complex problems (Guarnieri *et al.*, 1996, *Science*, 278, 361; Ouyang *et al.*, 1997, *Science*, 278, 446). Structural motifs of nucleic acid are emerging as unique tools in solving problems in nucleic acid computing (Sakamoto *et al.*, 2000, *Science*, 288, 1152). The use of nucleic acid sensor molecules that recognize specific sequence and structural information and that modulate this information are of value to nucleic acid computing.

In another embodiment, the invention features the use of nucleic acid sensor molecules in nucleic acid-based electronics utilized in nucleic acid computing applications. The combined use of nucleic acid sensor molecules in nucleic acid-based electronics and in nucleic acid computing bridges an important gap in the ability to generate signal output from nucleic acid-based computations. For example, the detection of specific nucleic acid sequences or structures that represent the solution to a problem or convey other information in a nucleic acid-based computation system by nucleic acid sensor molecules can result in the detection of a signal generated by a reporter molecule.

In one embodiment, the invention specifically features a process whereby a nucleic acid signaling molecule is used in a nucleic acid circuit. In response to a target signaling agent, for example current, the nucleic acid sensor molecule catalyzes a chemical reaction comprising ligation in response to a predetermined current or cleavage in response to a predetermined current. The nucleic acid circuit is thereby modulated between an open and a closed state based

on the predetermined input current that is applied to the circuit. A plurality of such circuits that comprise nucleic acid sensor modulation can be used in a variety of electronic devices, and can substitute solid state or silicon-based circuits in such devices. For example, computer processors comprising a plurality of nucleic acid sensor molecule based-circuits can be used in a computer device. Open and closed nucleic acid sensor molecule based-circuits can be used to generate or respond to binary code. Processing of nucleic acids by nucleic acid sensor molecules can be used to generate more complex code, for example where particular nucleic acid sequences represent different code variables.

Electronic devices comprising nucleic acid circuits which comprise nucleic acid sensor molecules of the invention are advantageous over the current state of the art circuits in terms of the absolute minimum size of the circuit and the device and the degree of modulation enabled by nucleic acid sensor molecules. For example, since nucleic acid sensor molecule-based circuits can be modulated by a variety of target signaling agents, modulation of the circuit is not limited to electronic signaling agents. Nucleic acid sensor molecule-based circuits can respond to biologic target signaling molecules, for example neurotransmitters, hormones, proteins, and nucleic acids, that enable the integration of the nucleic acid circuits into biological systems.

Several *in vitro* selection (evolution) strategies (Orgel, 1979, *Proc. R. Soc. London*, B 205, 435) have been used to evolve new nucleic acid catalysts capable of catalyzing cleavage and ligation of phosphodiester linkages (Joyce, 1989, *Gene*, 82, 83-87; Beaudry *et al.*, 1992, *Science* 257, 635-641; Joyce, 1992, *Scientific American* 267, 90-97; Breaker *et al.*, 1994, *TIBTECH* 12, 268; Bartel *et al.*, 1993, *Science* 261:1411-1418; Szostak, 1993, *TIBS* 17, 89-93; Kumar *et al.*, 1995, *FASEB J.*, 9, 1183; Breaker, 1996, *Curr. Op. Biotech.*, 7, 442; Santoro *et al.*, 1997, *Proc. Natl. Acad. Sci.*, 94, 4262; Tang *et al.*, 1997, *RNA* 3, 914; Nakamaye & Eckstein, 1994, *supra*; Long & Uhlenbeck, 1994, *supra*; Ishizaka *et al.*, 1995, *supra*; Vaish *et al.*, 1997, *Biochemistry* 36, 6495; Kuwabara *et al.*, 2000, *Curr. Opin. Chem. Biol.*, 4, 669) all of these are incorporated by reference herein). Each can catalyze a series of reactions including the hydrolysis of phosphodiester bonds in *trans* (and thus can cleave other RNA molecules) under physiological conditions.

There are several classes of enzymatic nucleic acids that are presently known. Each can catalyze the hydrolysis of RNA phosphodiester bonds in *trans* (and thus can cleave other RNA molecules) under physiological conditions. Table I summarizes some of the characteristics of a class of enzymatic nucleic acids known as ribozymes. In general, enzymatic nucleic acids act by first binding to a target. Such binding occurs, for example, through the interaction of the target RNA with one or more target binding portions of the enzymatic nucleic acid, wherein the target

RNA and substrate binding portion complex is held in close proximity to an enzymatic portion of the molecule that acts to cleave the target RNA. Thus, the enzymatic nucleic acid first recognizes and then binds a target RNA through complementary base-pairing, and once bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its function, such as its ability to direct synthesis of an encoded protein. After an enzymatic nucleic acid has bound and cleaved its RNA target, it is released from that RNA to search for another target and can repeatedly bind and cleave new targets. Thus, a single enzymatic nucleic acid molecule is able to cleave many molecules of target RNA. In addition, the enzymatic nucleic acid molecule is a highly specific inhibitor of gene expression, with the specificity of inhibition depending not only on the base-pairing mechanism of binding to the target RNA, but also on the mechanism of target RNA cleavage. Single mismatches, or base-substitutions, near the site of cleavage can completely eliminate catalytic activity of an enzymatic nucleic acid.

In one of the preferred embodiments of the inventions described herein, the nucleic acid sensor molecule is formed based on a hammerhead or hairpin motif. In other preferred embodiments, the nucleic acid sensor molecule is formed in the motif of a hepatitis delta virus, group I intron, group II intron or RNase P RNA (in association with an RNA guide sequence), *Neurospora* VS RNA, DNAzymes, NCH cleaving motifs, or G-cleavers. Examples of such hammerhead motifs are described by Dreyfus, *supra*, Rossi *et al.*, 1992, *AIDS Research and Human Retroviruses* 8, 183; of hairpin motifs by Hampel *et al.*, EP0360257, Hampel and Tritz, 1989 *Biochemistry* 28, 4929, Feldstein *et al.*, 1989, *Gene* 82, 53, Haseloff and Gerlach, 1989, *Gene*, 82, 43, and Hampel *et al.*, 1990 *Nucleic Acids Res.* 18, 299; Chowrira & McSwiggen, US. Patent No. 5,631,359; of the hepatitis delta virus motif is described by Perrotta and Been, 1992 *Biochemistry* 31, 16; of the RNase P motif by Guerrier-Takada *et al.*, 1983 *Cell* 35, 849; Forster and Altman, 1990, *Science* 249, 783; Li and Altman, 1996, *Nucleic Acids Res.* 24, 835; *Neurospora* VS RNA ribozyme motif is described by Collins (Saville and Collins, 1990 *Cell* 61, 685-696; Saville and Collins, 1991 *Proc. Natl. Acad. Sci. USA* 88, 8826-8830; Collins and Olive, 1993 *Biochemistry* 32, 2795-2799; Guo and Collins, 1995, *EMBO. J.* 14, 363); Group II introns are described by Griffin *et al.*, 1995, *Chem. Biol.* 2, 761; Michels and Pyle, 1995, *Biochemistry* 34, 2965; Pyle *et al.*, International PCT Publication No. WO 96/22689; of the Group I intron by Cech *et al.*, U.S. Patent 4,987,071 and of DNAzymes by Usman *et al.*, International PCT Publication No. WO 95/11304; Chartrand *et al.*, 1995, *NAR* 23, 4092; Breaker *et al.*, 1995, *Chem. Bio.* 2, 655; Santoro *et al.*, 1997, *PNAS* 94, 4262, and Beigelman *et al.*, International PCT publication No. WO 99/55857. NCH cleaving motifs are described in Ludwig & Sproat,

International PCT Publication No. WO 98/58058; and G-cleavers are described in Kore *et al.*, 1998, *Nucleic Acids Research* 26, 4116-4120 and Eckstein *et al.*, International PCT Publication No. WO 99/16871. Additional motifs such as the Aptazyme (aptamer dependent ribozyme) (Breaker *et al.*, WO 98/43993), Amberzyme (Class I motif; Figure 2; Beigelman *et al.*, U.S. Serial No. 09/301,511) and Zinzyme (Figure 3) (Beigelman *et al.*, U.S. Serial No. 09/301,511), all included by reference herein including drawings, can also be used in the present invention. These specific motifs are not limiting in the invention and those skilled in the art will recognize that all that is important in an nucleic acid sensor molecule with catalytic activity of this invention is that it has a specific substrate binding site which is complementary to one or more of the target nucleic acid regions, and that it have nucleotide sequences within or surrounding that substrate binding site which impart catalytic activity to the molecule (Cech *et al.*, U.S. Patent No. 4,987,071). Further, for nucleic acid sensor molecules, all that is important is that the molecule comprise sequence and structure that is able to interact with its substrate and catalyze a chemical reaction of interest.

Preferably, a nucleic acid molecule of the instant invention -is between 13 and 500 nucleotides in length. For example, nucleic acid sensor molecules of the invention are preferably between 25 and 300 nucleotides in length, more preferably between 30 and 150 nucleotides in length, *e.g.*, 34, 36, 38, 46, 47, 56, 65, 78, or 136 nucleotides in length. Exemplary DNAzymes of the invention are preferably between 15 and 400 nucleotides in length, more preferably between 25 and 150 nucleotides in length, *e.g.*, 29, 30, 31, or 32 nucleotides in length (see for example Santoro *et al.*, 1998, *Biochemistry*, 37, 13330-13342; Chartrand *et al.*, 1995, *Nucleic Acids Research*, 23, 4092-4096). Those skilled in the art will recognize that all that is required is for the nucleic acid molecule to be of length and conformation sufficient and suitable for the nucleic acid molecule to catalyze a reaction contemplated herein. The length of the nucleic acid molecules of the instant invention are not limiting within the general limits stated.

In a preferred embodiment, the invention provides a method for producing a class of nucleic acid-based diagnostic agents that exhibit a high degree of specificity for the target signaling molecule.

In additional embodiments, the invention features a method of detecting target signaling molecules or signaling agents in both *in vitro* and *in vivo* applications. *In vitro* diagnostic applications can comprise both solid support based and solution based chip, multichip-array, micro-well plate, and micro-bead derived applications as are commonly used in the art. *In vivo* diagnostic applications can include but are not limited to cell culture and animal model based



applications, comprising differential gene expression arrays, FACS based assays, diagnostic imaging, and others.

By "signaling agent" or "target signaling agent" is meant a chemical or physical entity capable of interacting with a nucleic acid sensor molecule, specifically a sensor component of a nucleic acid sensor molecule, resulting in modification of the enzymatic nucleic acid component of the nucleic acid sensor molecule via chemical, physical, topological, or conformational changes to the structure of the molecule such that the activity of the enzymatic nucleic acid component is modulated, for example is activated or deactivated. Signaling agents can comprise target signaling molecules such as macromolecules, ligands, small molecules, metals and ions, nucleic acid molecules including but not limited to RNA and DNA or analogs thereof, proteins, peptides, antibodies, polysaccharides, lipids, sugars, microbial or cellular metabolites, pharmaceuticals, and organic and inorganic molecules in a purified or unpurified form, or physical signals including magnetism, temperature, light, sound, shock, pH, capacitance, voltage, and ionic conditions.

By "enzymatic nucleic acid" is meant a nucleic acid molecule capable of catalyzing (altering the velocity and/or rate of) a variety of reactions including the ability to repeatedly cleave other separate nucleic acid molecules (endonuclease activity) or ligate other separate nucleic acid molecules (ligation activity) in a nucleotide base sequence-specific manner. Additional reactions amenable to nucleic acid sensor molecules include but are not limited to phosphorylation, dephosphorylation, isomerization, helicase activity, polymerization, transesterification, hydration, hydrolysis, alkylation, dealkylation, halogenation, dehalogenation, esterification, deesterification, hydrogenation, dehydrogenation, saponification, desaponification, amination, deamination, acylation, deacylation, glycosylation, deglycosylation, silation, desilation, hydroboration, epoxidation, peroxidation, carboxylation, decarboxylation, substitution, elimination, oxidation, and reduction reactions on both small molecules and macromolecules. Such a molecule with endonuclease and/or ligation activity can have complementarity in a substrate binding region to a specified gene target, and also has an enzymatic activity that specifically cleaves and/or ligates RNA or DNA in that target. That is, the nucleic acid molecule with endonuclease and/or ligation activity is able to intramolecularly or intermolecularly cleave and/or ligate RNA or DNA and thereby inactivate or activate a target RNA or DNA molecule. This complementarity functions to allow sufficient hybridization of the enzymatic RNA molecule to the target RNA or DNA to allow the cleavage/ligation to occur. 100% complementarity is preferred, but complementarity as low as 50-75% can also be useful in this invention. In addition, nucleic acid sensor molecule can perform other reactions, including

those mentioned above, selectively on both small molecule and macromolecular substrates, though specific interaction of the nucleic acid sensor molecule sequence with the desired substrate molecule via hydrogen bonding, electrostatic interactions, and Van der Waals interactions. The nucleic acids can be modified at the base, sugar, and/or phosphate groups. The term enzymatic nucleic acid is used interchangeably with phrases such as ribozymes, catalytic RNA, enzymatic RNA, catalytic DNA, catalytic oligonucleotides, nucleozyme, DNAzyme, RNA enzyme, endoribonuclease, endonuclease, minizyme, leadzyme, oligozyme, finderone or DNA enzyme. All of these terminologies describe nucleic acid molecules with enzymatic activity.

By "substrate binding arm" or "substrate binding domain" or "substrate binding region" is meant that portion or region of a nucleic acid sensor molecule which is able to interact, for example, via complementarity (*i.e.*, able to base-pair with), with a portion of its substrate. Preferably, such complementarity is 100%, but can be less if desired. For example, as few as 10 bases out of 14 can be base-paired (see for example Werner and Uhlenbeck, 1995, *Nucleic Acids Research*, 23, 2092-2096; Hammann *et al.*, 1999, *Antisense and Nucleic Acid Drug Dev.*, 9, 25-31). Examples of such arms are shown generally in Figures 1-4. That is, these arms contain sequences within a nucleic acid sensor molecule which are intended to bring the nucleic acid sensor molecule and the target signaling molecule, for example RNA, together through complementary base-pairing interactions. The nucleic acid sensor molecule of the invention can have binding arms that are contiguous or non-contiguous and can be of varying lengths. The length of the binding arm(s) are preferably greater than or equal to four nucleotides and of sufficient length to stably interact with the target RNA. Preferably, the binding arm(s) are 12-100 nucleotides in length. More preferably, the binding arms are 14-24 nucleotides in length (see, for example, Werner and Uhlenbeck, *supra*; Hamman *et al.*, *supra*; Hampel *et al.*, EP0360257; Berzal-Herrance *et al.*, 1993, *EMBO J.*, 12, 2567-73). If two binding arms are chosen, the design is such that the length of the binding arms are symmetrical (*i.e.*, each of the binding arms is of the same length; *e.g.*, five and five nucleotides, or six and six nucleotides, or seven and seven nucleotides long) or asymmetrical (*i.e.*, the binding arms are of different length; *e.g.*, six and three nucleotides; three and six nucleotides long; four and five nucleotides long; four and six nucleotides long; four and seven nucleotides long; and the like).

By "enzymatic portion" or "catalytic domain" is meant that portion or region of the nucleic acid sensor molecule essential for catalyzing a chemical reaction, such as cleavage of a nucleic acid substrate.

By "system" is meant, material, in a purified or unpurified form, from biological or non-biological sources, including but not limited to human, animal, plant, bacteria, virus, fungi, soil,

water, mechanical devices, circuits, networks, computers, or others that comprises the target signaling agent or target signaling molecule to be detected or amplified.

The "biological system" as used herein can be a eukaryotic system or a prokaryotic system, for example a bacterial cell, plant cell or a mammalian cell, or of plant origin, mammalian origin, yeast origin, *Drosophila* origin, or archebacterial origin.

By "reporter molecule" is meant a molecule, such as a nucleic acid sequence (e.g., RNA or DNA or analogs thereof) or peptides and/or other chemical moieties, able to stably interact with the nucleic acid sensor molecule and function as a substrate for the nucleic acid sensor molecule. The reporter molecule can also contain chemical moieties capable of generating a detectable response, including but not limited to, fluorescent, chromogenic, radioactive, enzymatic and/or chemiluminescent or other detectable labels that can then be detected using standard assays known in the art. The reporter molecule can also act as an intermediate in a chain of events, for example, by acting as an amplicon, inducer, promoter, or inhibitor of other events that can act as second messengers in a system.

In one embodiment, the reporter molecule of the invention is an oligonucleotide primer, template, or probe, which can be used to modulate the amplification of additional nucleic acid sequences, for example, sequences comprising reporter molecules, target signaling molecules, effector molecules, inhibitor molecules, and/or additional nucleic acid sensor molecules of the instant invention.

By "sensor component" of the nucleic acid sensor molecule is meant, a molecule such as a nucleic acid sequence (e.g., RNA or DNA or analogs thereof), peptide, or other chemical moiety which can interact with one or more regions of the enzymatic nucleic acid component of the nucleic acid sensor molecule to modulate, such as inhibit or activate, the catalytic activity of the nucleic acid sensor molecule. In the presence of a signaling agent, the ability of the sensor component, for example, to modulate the catalytic activity of the enzymatic nucleic acid component is inhibited or diminished. The sensor component can comprise recognition properties relating to chemical or physical signals capable of modulating the enzymatic nucleic acid component via chemical or physical changes to the structure of the nucleic acid sensor molecule. The sensor component can be covalently linked to the nucleic acid sensor molecule, or can be non-covalently associated. A person skilled in the art will recognize that all that is required is that the sensor component is able to selectively inhibit the activity of the nucleic acid sensor molecule.

"Complementarity" refers to the ability of a nucleic acid to form hydrogen bond(s) with another RNA sequence by either traditional Watson-Crick or other non-traditional types. In

reference to the nucleic molecules of the present invention, the binding free energy for a nucleic acid molecule with its target or complementary sequence is sufficient to allow the relevant function of the nucleic acid to proceed, e.g., enzymatic nucleic acid cleavage, ligation, isomerization, phosphorylation, or dephosphorylation. Determination of binding free energies for nucleic acid molecules is well known in the art (see, e.g., Turner et al., 1987, *CSH Symp. Quant. Biol.* LII pp.123-133; Frier et al., 1986, *Proc. Nat. Acad. Sci. USA* 83:9373-9377; Turner et al., 1987, *J. Am. Chem. Soc.* 109:3783-3785). A percent complementarity indicates the percentage of contiguous residues in a nucleic acid molecule which can form hydrogen bonds (e.g., Watson-Crick base pairing) with a second nucleic acid sequence (e.g., 5, 6, 7, 8, 9, 10 out of 10 being 50%, 60%, 70%, 80%, 90%, and 100% complementary). "Perfectly complementary" means that all the contiguous residues of a nucleic acid sequence will hydrogen bond with the same number of contiguous residues in a second nucleic acid sequence.

By "alkyl" group is meant a saturated aliphatic hydrocarbon, including straight-chain, branched-chain, and cyclic alkyl groups. Preferably, the alkyl group has 1 to 12 carbons. More preferably it is a lower alkyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkyl group can be substituted or unsubstituted. When substituted the substituted group(s) are preferably, hydroxyl, cyano, alkoxy, =O, =S, NO<sub>2</sub> or N(CH<sub>3</sub>)<sub>2</sub>, amino, or SH. The term also includes alkenyl groups which are unsaturated hydrocarbon groups containing at least one carbon-carbon double bond, including straight-chain, branched-chain, and cyclic groups. Preferably, the alkenyl group has 1 to 12 carbons. More preferably it is a lower alkenyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkenyl group can be substituted or unsubstituted. When substituted the substituted group(s) can be preferably, hydroxyl, cyano, alkoxy, =O, =S, NO<sub>2</sub>, halogen, N(CH<sub>3</sub>)<sub>2</sub>, amino, or SH. The term "alkyl" also includes alkynyl groups which have an unsaturated hydrocarbon group containing at least one carbon-carbon triple bond, including straight-chain, branched-chain, and cyclic groups. Preferably, the alkynyl group has 1 to 12 carbons. More preferably it is a lower alkynyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkynyl group can be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, cyano, alkoxy, =O, =S, NO<sub>2</sub> or N(CH<sub>3</sub>)<sub>2</sub>, amino or SH. Such alkyl groups can also include aryl, alkylaryl, carbocyclic aryl, heterocyclic aryl, amide and ester groups. An "aryl" group refers to an aromatic group which has at least one ring having a conjugated p electron system and includes carbocyclic aryl, heterocyclic aryl and biaryl groups, all of which can be optionally substituted. The preferred substituent(s) of aryl groups are halogen, trihalomethyl, hydroxyl, SH, OH, cyano, alkoxy, alkyl, alkenyl, alkynyl, and amino groups. An "alkylaryl" group refers to an alkyl group (as described

above) covalently joined to an aryl group (as described above). Carbocyclic aryl groups are groups wherein the ring atoms on the aromatic ring are all carbon atoms. The carbon atoms are optionally substituted. Heterocyclic aryl groups are groups having from 1 to 3 heteroatoms as ring atoms in the aromatic ring and the remainder of the ring atoms are carbon atoms. Suitable heteroatoms include oxygen, sulfur, and nitrogen, and include furanyl, thienyl, pyridyl, pyrrolyl, N-lower alkyl pyrrolo, pyrimidyl, pyrazinyl, imidazolyl and the like, all optionally substituted. An "amide" refers to an -C(O)-NH-R, where R is either alkyl, aryl, alkylaryl or hydrogen. An "ester" refers to an -C(O)-OR', where R is either alkyl, aryl, alkylaryl or hydrogen.

By "nucleotide" is meant a heterocyclic nitrogenous base in N-glycosidic linkage with a phosphorylated sugar. Nucleotides are recognized in the art to include natural bases (standard), and modified bases well known in the art. Such bases are generally located at the 1' position of a nucleotide sugar moiety. Nucleotides generally comprise a base, sugar and a phosphate group. The nucleotides can be unmodified or modified at the sugar, phosphate and/or base moiety, (also referred to interchangeably as nucleotide analogs, modified nucleotides, non-natural nucleotides, non-standard nucleotides and other; see for example, Usman and McSwiggen, *supra*; Eckstein *et al.*, International PCT Publication No. WO 92/07065; Usman *et al.*, International PCT Publication No. WO 93/15187; Uhlman & Peyman, *supra* all are hereby incorporated by reference herein). There are several examples of modified nucleic acid bases known in the art as summarized by Limbach *et al.*, 1994, *Nucleic Acids Res.* 22, 2183. Some of the non-limiting examples of chemically modified and other natural nucleic acid bases that can be introduced into nucleic acids include, inosine, purine, pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 2,4,6-trimethoxy benzene, 3-methyl uracil, dihydrouridine, naphthyl, aminophenyl, 5-alkylcytidines (e.g., 5-methylcytidine), 5-alkyluridines (e.g., ribothymidine), 5-halouridine (e.g., 5-bromouridine) or 6-azapyrimidines or 6-alkylpyrimidines (e.g. 6-methyluridine), propyne, quesosine, 2-thiouridine, 4-thiouridine, wybutosine, wybutoxosine, 4-acetylcytidine, 5-(carboxyhydroxymethyl)uridine, 5'-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluridine, beta-D-galactosylqueosine, 1-methyladenosine, 1-methylinosine, 2,2-dimethylguanosine, 3-methylcytidine, 2-methyladenosine, 2-methylguanosine, N6-methyladenosine, 7-methylguanosine, 5-methoxyaminomethyl-2-thiouridine, 5-methylaminomethyluridine, 5-methylcarbonylmethyluridine, 5-methoxyuridine, 5-methyl-2-thiouridine, 2-methylthio-N6-isopentenyladenosine, beta-D-mannosylqueosine, uridine-5-oxyacetic acid, 2-thiocytidine, threonine derivatives and others (Burgin *et al.*, 1996, *Biochemistry*, 35, 14090; Uhlman & Peyman, *supra*). By "modified bases" in this aspect is meant nucleotide bases other than adenine, guanine, cytosine and uracil at 1' position or their

equivalents; such bases can be used at any position, for example, within the catalytic core of an nucleic acid sensor molecule and/or in the substrate-binding regions of the nucleic acid molecule.

By "nucleoside" is meant a heterocyclic nitrogenous base in N-glycosidic linkage with a sugar. Nucleosides are recognized in the art to include natural bases (standard), and modified bases well known in the art. Such bases are generally located at the 1' position of a nucleoside sugar moiety. Nucleosides generally comprise a base and sugar group. The nucleosides can be unmodified or modified at the sugar, and/or base moiety, (also referred to interchangeably as nucleoside analogs, modified nucleosides, non-natural nucleosides, non-standard nucleosides and other; see for example, Usman and McSwiggen, *supra*; Eckstein *et al.*, International PCT Publication No. WO 92/07065; Usman *et al.*, International PCT Publication No. WO 93/15187; Uhlman & Peyman, *supra* all are hereby incorporated by reference herein). There are several examples of modified nucleic acid bases known in the art as summarized by Limbach *et al.*, 1994, *Nucleic Acids Res.* 22, 2183. Some of the non-limiting examples of chemically modified and other natural nucleic acid bases that can be introduced into nucleic acids include, inosine, purine, pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 2,4,6-trimethoxy benzene, 3-methyl uracil, dihydrouridine, naphthyl, aminophenyl, 5-alkylcytidines (*e.g.*, 5-methylcytidine), 5-alkyluridines (*e.g.*, ribothymidine), 5-halouridine (*e.g.*, 5-bromouridine) or 6-azapyrimidines or 6-alkylpyrimidines (*e.g.* 6-methyluridine), propyne, queosine, 2-thiouridine, 4-thiouridine, wybutosine, wybutoxosine, 4-acetylcytidine, 5-(carboxyhydroxymethyl)uridine, 5'-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluridine, beta-D-galactosylqueosine, 1-methyladenosine, 1-methylinosine, 2,2-dimethylguanosine, 3-methylcytidine, 2-methyladenosine, 2-methylguanosine, N6-methyladenosine, 7-methylguanosine, 5-methoxyaminomethyl-2-thiouridine, 5-methylaminomethyluridine, 5-methylcarbonylmethyluridine, 5-methyloxyuridine, 5-methyl-2-thiouridine, 2-methylthio-N6-isopentenyladenosine, beta-D-mannosylqueosine, uridine-5-oxyacetic acid, 2-thiocytidine, threonine derivatives and others (Burgin *et al.*, 1996, *Biochemistry*, 35, 14090; Uhlman & Peyman, *supra*). By "modified bases" in this aspect is meant nucleoside bases other than adenine, guanine, cytosine and uracil at 1' position or their equivalents; such bases can be used at any position, for example, within the catalytic core of an nucleic acid sensor molecule and/or in the substrate-binding regions of the nucleic acid molecule.

By "unmodified nucleotide" is meant a nucleotide with one of the bases adenine, cytosine, guanine, thymine, uracil joined to the 1' carbon of  $\square$  beta-D-ribo-furanose.

By "modified nucleotide" is meant a nucleotide that contains a modification in the chemical structure of an unmodified nucleotide base, sugar and/or phosphate.

By "unmodified nucleoside" is meant a nucleoside with one of the bases adenine, cytosine, guanine, thymine, uracil joined to the 1' carbon of  $\beta$ -D-ribo-furanose.

By "modified nucleoside" is meant a nucleotide that contains a modification in the chemical structure of an unmodified nucleoside base or sugar.

By "Inozyme" or "NCH" motif is meant, an enzymatic nucleic acid molecule comprising a motif as is generally described as NCH Rz in Figure 1. Inozymes possess endonuclease activity to cleave RNA substrates having a cleavage triplet NCH/, where N is a nucleotide, C is cytidine and H is adenosine, uridine or cytidine, and / represents the cleavage site. H is used interchangeably with X. Inozymes can also possess endonuclease activity to cleave RNA substrates having a cleavage triplet NCN/, where N is a nucleotide, C is cytidine, and / represents the cleavage site. "T" in Figure 1 represents an Inosine nucleotide, preferably a ribo-Inosine or xylo-Inosine nucleoside

By "G-cleaver" motif is meant, an enzymatic nucleic acid molecule comprising a motif as is generally described as G-cleaver Rz in Figure 1. G-cleavers possess endonuclease activity to cleave RNA substrates having a cleavage triplet NYN/, where N is a nucleotide, Y is uridine or cytidine and / represents the cleavage site. G-cleavers can be chemically modified as is generally shown in Figure 1.

By "amberzyme" motif is meant, an enzymatic nucleic acid molecule comprising a motif as is generally described in Figure 2. Amberzymes possess endonuclease activity to cleave RNA substrates having a cleavage triplet NG/N, where N is a nucleotide, G is guanosine, and / represents the cleavage site. Amberzymes can be chemically modified to increase nuclease stability through substitutions as are generally shown in Figure 2. In addition, differing nucleoside and/or non-nucleoside linkers can be used to substitute the 5'-gaaa-3' loops shown in the figure. Amberzymes represent a non-limiting example of an enzymatic nucleic acid molecule that does not require a ribonucleotide (2'-OH) group within its own nucleic acid sequence for activity.

By "zinzyme" motif is meant, an enzymatic nucleic acid molecule comprising a motif as is generally described in Figure 3. Zinzymes possess endonuclease activity to cleave RNA substrates having a cleavage triplet including but not limited to YG/Y, where Y is uridine or cytidine, and G is guanosine and / represents the cleavage site. Zinzymes can be chemically modified to increase nuclease stability through substitutions as are generally shown in Figure 3, including substituting 2'-O-methyl guanosine nucleotides for guanosine nucleotides. In addition,

differing nucleotide and/or non-nucleotide linkers can be used to substitute the 5'-gaaa-2' loop shown in the figure. Zinzymes represent a non-limiting example of an enzymatic nucleic acid molecule that does not require a ribonucleotide (2'-OH) group within its own nucleic acid sequence for activity.

By "DNAzyme" is meant, an enzymatic nucleic acid molecule that does not require the presence of a 2'-OH group within it for its activity. In particular embodiments the enzymatic nucleic acid molecule can have an attached linker(s) or other attached or associated groups, moieties, or chains containing one or more nucleotides with 2'-OH groups. DNAzymes can be synthesized chemically or expressed endogenously *in vivo*, by means of a single stranded DNA vector or equivalent thereof. An example of a DNAzyme is shown in Figure 4 and is generally reviewed in Usman *et al.*, International PCT Publication No. WO 95/11304; Chartrand *et al.*, 1995, *NAR* 23, 4092; Breaker *et al.*, 1995, *Chem. Bio.* 2, 655; Santoro *et al.*, 1997, *PNAS* 94, 4262; Breaker, 1999, *Nature Biotechnology*, 17, 422-423; and Santoro *et al.*, 2000, *J. Am. Chem. Soc.*, 122, 2433-39; Perrin *et al.*, 2001, *JACS*, 123, 1556. Additional DNAzyme motifs can be selected for using techniques similar to those described in these references, and hence, are within the scope of the present invention.

By "sufficient length" is meant an oligonucleotide of greater than or equal to 3 nucleotides in length and long enough to provide the intended function (such as binding) under the expected condition. For example, for binding arms of a nucleic acid sensor molecule, "sufficient length" means that the binding arm sequence is long enough to provide stable binding to a target site under the expected reaction conditions and environment. Preferably, the binding arms are not so long as to prevent useful turnover of the nucleic acid molecule

By "stably interact" is meant interaction of the oligonucleotides with target nucleic acid (e.g., by forming hydrogen bonds with complementary nucleotides in the target under physiological conditions) that is sufficient to the intended purpose (e.g., cleavage of target RNA by an enzyme).

By "nucleic acid molecule" as used herein is meant a molecule comprising nucleotides. The nucleic acid can be single, double, or multiple stranded and can comprise modified or unmodified nucleotides or non-nucleotides or various mixtures and combinations thereof. Nucleic acid molecules shall include oligonucleotides, ribozymes, DNAzymes, templates, and primers.

By "oligonucleotide" is meant a nucleic acid molecule comprising a stretch of three or more nucleotides.



In a preferred embodiment the linker region, when present in the nucleic acid sensor molecule and/or reporter molecule is further comprised of nucleotide, non-nucleotide chemical moieties or combinations thereof. Non-limiting examples of non-nucleotide chemical moieties can include ester, anhydride, amide, nitrile, and/or phosphate groups.

In another embodiment, the non-nucleotide linker is as defined herein. The term "non-nucleotide" as used herein include either abasic nucleotide, polyether, polyamine, polyamide, peptide, carbohydrate, lipid, or polyhydrocarbon compounds. Specific examples include those described by Seela and Kaiser, *Nucleic Acids Res.* 1990, 18:6353 and *Nucleic Acids Res.* 1987, 15:3113; Cload and Schepartz, *J. Am. Chem. Soc.* 1991, 113:6324; Richardson and Schepartz, *J. Am. Chem. Soc.* 1991, 113:5109; Ma et al., *Nucleic Acids Res.* 1993, 21:2585 and *Biochemistry* 1993, 32:1751; Durand et al., *Nucleic Acids Res.* 1990, 18:6353; McCurdy et al., *Nucleosides & Nucleotides* 1991, 10:287; Jschke et al., *Tetrahedron Lett.* 1993, 34:301; Ono et al., *Biochemistry* 1991, 30:9914; Arnold et al., International Publication No. WO89/02439; Usman et al., International Publication No. WO 95/06731; Dudycz et al., International Publication No. WO 95/11910 and Ferentz and Verdine, *J. Am. Chem. Soc.* 1991, 113:4000, all hereby incorporated by reference herein. Thus, in a preferred embodiment, the invention features an nucleic acid sensor molecule of the invention having one or more non-nucleotide moieties, and having enzymatic activity to perform a chemical reaction, for example to cleave an RNA or DNA molecule.

By "cap structure" is meant chemical modifications which have been incorporated at either terminus of the oligonucleotide (see for example Wincott et al., WO 97/26270, incorporated by reference herein). These terminal modifications protect the nucleic acid molecule from exonuclease degradation, and can help in delivery and/or localization within a cell. The cap can be present at the 5'-terminus (5'-cap) or at the 3'-terminus (3'-cap) or can be present on both termini. In non-limiting examples: the 5'-cap is selected from the group comprising inverted abasic residue (moiety), 4',5'-methylene nucleotide; 1-(beta-D-erythrofuransyl) nucleotide, 4'-thio nucleotide, carbocyclic nucleotide; 1,5-anhydrohexitol nucleotide; L-nucleotides; alpha-nucleotides; modified base nucleotide; phosphorodithioate linkage; *threo*-pentofuransyl nucleotide; acyclic 3',4'-seco nucleotide; acyclic 3,4-dihydroxybutyl nucleotide; acyclic 3,5-dihydroxypentyl nucleotide, 3'-3'-inverted nucleotide moiety; 3'-3'-inverted abasic moiety; 3'-2'-inverted nucleotide moiety; 3'-2'-inverted abasic moiety; 1,4-butanediol phosphate; 3'-phosphoramidate; hexylphosphate; aminohexyl phosphate; 3'-phosphate; 3'-phosphorothioate; phosphorodithioate; or bridging or non-bridging methylphosphonate moiety (for more details see Wincott et al., International PCT publication No. WO 97/26270, incorporated by reference

herein). In yet another preferred embodiment the 3'-cap is selected from a group comprising, 4',5'-methylene nucleotide; 1-(beta-D-erythrofuransyl) nucleotide; 4'-thio nucleotide, carbocyclic nucleotide; 5'-amino-alkyl phosphate; 1,3-diamino-2-propyl phosphate, 3-aminopropyl phosphate; 6-aminoethyl phosphate; 1,2-aminododecyl phosphate; hydroxypropyl phosphate; 1,5-anhydrohexitol nucleotide; L-nucleotide; alpha-nucleotide; modified base nucleotide; phosphorodithioate; *threo*-pentofuransyl nucleotide; acyclic 3',4'-seco nucleotide; 3,4-dihydroxybutyl nucleotide; 3,5-dihydroxypentyl nucleotide, 5'-5'-inverted nucleotide moiety; 5'-5'-inverted abasic moiety; 5'-phosphoramidate; 5'-phosphorothioate; 1,4-butanediol phosphate; 5'-amino; bridging and/or non-bridging 5'-phosphoramidate, phosphorothioate and/or phosphorodithioate, bridging or non bridging methylphosphonate and 5'-mercapto moieties (for more details see Beaucage and Iyer, 1993, *Tetrahedron* 49, 1925; incorporated by reference herein).

By "abasic" or "abasic nucleotide" is meant sugar moieties lacking a base or having other chemical groups in place of a base at the 1' position, (for more details see Wincott *et al.*, International PCT publication No. WO 97/26270).

The term "non-nucleotide" refers to any group or compound which can be incorporated into a nucleic acid chain in the place of one or more nucleotide units, including either sugar and/or phosphate substitutions, and allows the remaining bases to exhibit their enzymatic activity. The group or compound is abasic in that it does not contain a commonly recognized nucleotide base, such as adenine, guanine, cytosine, uracil or thymine. The terms "abasic" or "abasic nucleotide" are meant to include sugar moieties lacking a base or having other chemical groups in place of a base at the 1' position, (for more details see Wincott *et al.*, International PCT publication No. WO 97/26270).

By "RNA" is meant a molecule comprising at least one ribonucleotide residue. By "ribonucleotide" or "2'-OH" is meant a nucleotide with a hydroxyl group at the 2' position of a  $\beta$ -D-ribo-furanose moiety.

By "patient" is meant an organism, which is a donor or recipient of explanted cells or the cells themselves. "Patient" also refers to an organism to which the nucleic acid molecules of the invention can be administered. Preferably, a patient is a mammal or mammalian cells. More preferably, a patient is a human or human cells.

By "enhanced enzymatic activity" is meant to include activity measured in cells and/or *in vivo* where the activity is a reflection of both the catalytic activity and the stability of the nucleic acid molecules of the invention. In this invention, the product of these properties can be increased *in vivo* compared to an all RNA enzymatic nucleic acid or all DNA enzyme. In some

cases, the individual catalytic activity or stability of the nucleic acid molecule can be decreased (i.e., less than ten-fold), but the overall activity of the nucleic acid molecule is enhanced, *in vivo*.

By "nucleic acid circuit" or "nucleic acid-based circuit" is meant an electronic circuit comprising one or more nucleic acids or oligonucleotides.

By "nucleic acid computer" or "nucleic acid-based computer" is meant a computing device or system comprising one or more nucleic acids or oligonucleotides. The nucleic acid computer can be used to interface biological systems, control other devices, or can be utilized to solve problems and/or manipulate data. Furthermore, the nucleic acid computer may comprise nucleic acid circuits.

By "comprising" is meant including, but not limited to, whatever follows the word "comprising". Thus, use of the term "comprising" indicates that the listed elements are required or mandatory, but that other elements are optional and can or can not be present. By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of". Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements can be present.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

#### Description Of The Preferred Embodiments

The drawings will first briefly be described.

##### Drawings:

Figure 1 shows examples of chemically stabilized ribozyme motifs. **HH Rz**, represents hammerhead ribozyme motif (Usman *et al.*, 1996, *Curr. Op. Struct. Bio.*, 1, 527); **NCH Rz** represents the NCH ribozyme motif (Ludwig & Sproat, International PCT Publication No. WO 98/58058); **G-Cleaver**, represents G-cleaver ribozyme motif (Kore *et al.*, 1998, *Nucleic Acids Research* 26, 4116-4120). **N** or **n**, represent independently a nucleotide which can be same or different and have complementarity to each other; **rI**, represents ribo-Inosine nucleotide; arrow indicates the site of cleavage within the target. Position 4 of the HH Rz and the NCH Rz is shown as having 2'-C-allyl modification, but those skilled in the art will recognize that this position can be modified with other modifications well known in the art, so long as such modifications do not significantly inhibit the activity of the ribozyme.

Figure 2 shows an example of the Amberzyme ribozyme motif that is chemically stabilized (see, for example, Beigelman *et al.*, International PCT publication No. WO 99/55857,

incorporated by reference herein; also referred to as Class I Motif). The Amberzyme motif is a class of enzymatic nucleic molecules that do not require the presence of a ribonucleotide (2'-OH) group for its activity.

Figure 3 shows an example of the Zinzyme A ribozyme motif that is chemically stabilized (Beigelman *et al.*, International PCT publication No. WO 99/55857, incorporated by reference herein; also referred to as Class A or Class II Motif). The Zinzyme motif is a class of enzymatic nucleic molecules that do not require the presence of a ribonucleotide (2'-OH) group for its activity.

Figure 4 shows an example of a DNAzyme motif described by Santoro *et al.*, 1997, *PNAS*, 94, 4262.

Figure 5 shows a non-limiting example of the detection of a target sequence using a hammerhead-based cis-blocking sequence strategy. In this case, the enzymatic nucleic acid component is the nucleic acid sensor molecule, and in the absence of target, is inactivated by intramolecular folding. Addition of target sequence allows interaction of the sensor molecule/target complex to the reporter sequence to allow cleavage of the reporter molecule by the activated target/sensor molecule complex, providing a fluorescent signal due to the separation of fluorophore and quench molecules. This same concept can be applied to other enzymatic nucleic acid components of the instant invention, including but not limited to Inozymes, G-cleavers, DNAzymes, Zinzymes, Amberzymes, and Hairpins. In addition, the configuration of the blocking sequence can hybridize with a variety of sequence positions both in *cis* and in *trans* (e.g., intermolecular binding and/or intramolecular binding) and in a variety of different locations on the sensor molecule. Additional non-limiting configurations are summarized in Figures 7-9.

Figure 6 shows a schematic diagram indicating the two primary configurations of a cis-acting nucleic acid sensor molecule. The molecule can be either bound to a target sequence (A) or unbound and therefore bound to itself (B).

Figure 7 displays a number of potential secondary structures for the nucleic acid sensor molecules in non-limiting examples. Schemes A-D show the activation of a nucleic acid sensor molecule by target sequence binding to a sensor component of the nucleic acid sensor molecule enabling catalysis, for example cleavage, of a reporter molecule.

Figure 8 displays a number of potential secondary structures for the nucleic acid sensor molecules in non-limiting examples. Schemes E-H show the activation of a nucleic acid sensor molecule by target sequence binding to a sensor component of the nucleic acid sensor molecule enabling catalysis, for example cleavage, of a reporter molecule.

Figure 9 displays a number of potential secondary structures for the nucleic acid sensor molecules in non-limiting examples. Schemes I and J show the activation of a nucleic acid sensor molecule by target sequence binding to a sensor component of the nucleic acid sensor molecule enabling catalysis, for example cleavage, of a reporter molecule.

Figure 10 displays a number of potential secondary structures for the nucleic acid sensor molecules in non-limiting examples. Scheme K shows the activation of a nucleic acid sensor molecule by target sequence binding to a sensor component of the nucleic acid sensor molecule enabling catalysis, for example cleavage, of a reporter molecule.

Figure 11 displays a number of potential secondary structures for the nucleic acid sensor molecules in non-limiting examples. Schemes M and N show the activation of a nucleic acid sensor molecule by target sequence binding to a peptide sensor component of the nucleic acid sensor molecule enabling catalysis, for example cleavage, of a reporter molecule.

Figure 12 displays a number of potential secondary structures for the nucleic acid sensor molecules in non-limiting examples. Schemes O and P show the activation of a nucleic acid sensor molecule by target sequence binding to a sensor component of the nucleic acid sensor molecule enabling catalysis, for example cleavage, of a reporter molecule.

Figure 13 displays a number of potential secondary structures for the nucleic acid sensor molecules in non-limiting examples. Schemes Q and R show the activation of a nucleic acid sensor molecule by target sequence binding to a sensor component of the nucleic acid sensor molecule enabling catalysis, for example cleavage, of a reporter molecule.

Figure 14 displays the inherent amplification capacity of the diagnostic system of the instant invention. Due to the catalytic nature of the nucleic acid sensor molecule, catalysis can take place on a multitude of reporter molecules, resulting in an amplified signal in response to a single target signaling molecule.

Figure 15 shows the structure of a diagnostic system of the instant invention, comprising a nucleic acid sensor molecule and a separate sensor component.

Figure 16 is a bar graph that shows the results of testing nucleic acid sensor molecule/sensor component combinations in a cleavage assay. The reporter molecules were 5'-end labeled with  $^{32}\text{P}$ -phosphate and incubated for 12 or 60 minutes in either: (1) buffer alone (50 mM Tris, pH 7.5, 10 mM  $\text{MgCl}_2$ ), or in the presence of (2) 10 nM nucleic acid sensor molecule, (3) 10 nM nucleic acid sensor molecule plus 20 nM sensor component, (4) 10 nM nucleic acid sensor molecule plus 200 nM sensor component, or (5) 10 nM nucleic acid sensor molecule plus 20 nM sensor component and 500 nM target signaling molecule. At the end of the incubation the reactions were loaded onto a PAGE gel to separate cleaved reporter from uncleaved reporter.

The gel was imaged on a Molecular Dynamics phosphorimager and quantitated to determine the percent of reporter cleaved under each set of conditions. Control reactions were carried out to ensure that addition of sensor component or target signaling molecule sequence, without nucleic acid sensor molecule, did not result in reporter cleavage; only 0.2-0.4% of reporter was cleaved under these conditions.

Figures 17a-c are a schematic representation of the method of the invention used to isolate nucleic acid sensor molecules capable of autoligation reactions useful in a variety of applications, including diagnostic applications. Figure 17a shows the general selection scheme used for isolating active sequences. A random pool of nucleic acid sequence, such as RNA is combined with a substrate molecule comprising the structure R1-L-R2-Biotin, wherein R1 is selected from the group consisting of methyl, hydrogen, phosphate, nucleoside, nucleotide, oligonucleotide, R2 is selected from the group comprising molecules capable of generating a detectable signal, such as molecular beacons, small molecules, fluorophores, chemophores, ionophores, radio-isotopes, photophores, peptides, proteins, enzymes, antibodies, nucleic acids, and enzymatic nucleic acids, L represents a linker which can be present or absent, and "-" represents a covalent bond. Catalytically active sequences are biotinylated. The reaction mixture is passed over a solid support derivatized with Avidin, resulting in the capture of the biotinylated, catalytically active sequence pool. The support bound sequences are amplified by methods known in the art. Figure 17b shows the selection of the initial pool of sequences that provide ligation activity, and subsequent selection of molecules that are active in the presence of a ligand of diagnostic interest. Initially, selection of catalytic sequences takes place in the absence of the ligand of diagnostic interest. The active molecules isolated from the first round of selection that initially bind to the Avidin derivatized support are eliminated. Molecules that pass through the support are re-selected in the presence of the ligand of diagnostic interest. The re-selected pool that binds to the support after reaction in the presence of the ligand of diagnostic interest is amplified by methods known in the art and transcribed for subsequent rounds of selection. Figure 17c shows another selection strategy for isolating nucleic acid molecules capable of autoligation in the presence of a ligand of diagnostic interest. In this case, an initial selection takes place in the absence of the ligand to select sequences with autoligation activity. This pool is mutagenized by methods known in the art. The resulting mutagenized pool is selected for ligand binding activity by methods known in the art, for example, by using ligand affinity chromatography or gel shift assays. The resulting pool is mutagenized by methods known in the art. The original selection (for activity) is repeated in the presence of the ligand of diagnostic interest, with counterselection for molecules that react in the absence of the ligand.

Figures 18a-c are a schematic representation of the method of the invention used to isolate nucleic acid sensor molecules capable of isomerization reactions that have applications in a variety of fields, including diagnostics. R1 and R2 represent compounds, which can be the same or different, capable of generating a detectable signal or quenching a detectable signal when an isomerization event takes place, comprising molecular beacons, small molecules, fluorophores, chemophores, ionophores, radio-isotopes, photophores, peptides, proteins, enzymes, antibodies, nucleic acids, and enzymatic nucleic acids, L represents a linker which can be present or absent, and “-” represents a covalent bond. Figure 18a shows the general selection scheme used for isolating active sequences. A random pool of nucleic acid sequences are passed over the complex of interest, derivatized to a solid support. The representative example of the complex shown in the figure consists of two fluorescent molecules joined together via a cis-carbon double bond linkage. Alternatively, a trans-carbon double bond linkage can be used. The selection pool is enriched and mutagenized throughout multiple generations to generate a diverse pool of “cis” binding sequences. Cis-binding nucleic acid molecules are then loaded onto the resin and the corresponding trans isomer of the complex is used to elute sequences that bind the trans-isomer tighter than the cis-isomer. Figure 18b shows how the concentration of cis-isomer on the resin and the concentration of trans-isomer eluant can be manipulated in order to select sequences that prefer binding to one isomer over the other, and can therefor drive the reaction in the desired direction. Figure 18c shows a selection scheme for isolating signaling agent dependent nucleic acid isomerase molecules from the initial selection pool from Figure 18a. A counter-selection takes place in which sequences that are bound to the cis-isomer complex are eluted with a signaling agent of interest. An additional counter-selection takes place in which sequences that are bound to the cis-isomer complex are eluted with the signaling agent of interest. A selection then takes place in which sequences remaining from the counter-selection rounds that are bound to the cis-isomer complex are eluted with a mixture of the signaling agent of interest and the trans-isomer complex, the eluted ligand dependent nucleic acid catalyst sequences are amplified and transcribed by methods known in the art.

Figure 19 shows non-limiting examples of a Zinzyme sensor molecule. In the example provided, in the presence of a target signaling molecule (SEQ ID NO. 25), for example the stem-loop III region of Hepatitis C virus (HCV) (SEQ ID NO. 26), the sensor molecule adopts a conformation that cleaves a tagged reporter molecule, for example *in trans* (SEQ ID NO. 22) providing cleave of the reporter molecule at 5'-NYG/N-3', where / represents the cleavage site, N represents any nucleotide, Y represents any pyrimidine nucleotide, and G represents Guanosine. Alternatively, a sensor molecule/reporter molecule complex, for example (SEQ ID

NO. 27), provides cleavage of a tagged-reporter molecule *in cis*, resulting in the release of the Tag, for example Tag-AGAAC. The Tag can comprise beacons, small molecules, fluorophores, chemophores, ionophores, radio-isotopes, photophores, peptides, proteins, enzymes, antibodies, nucleic acids, and enzymatic nucleic acids.

Figure 20 shows non-limiting examples of Zinzyme sensor molecules. In the examples provided, in the presence of a target protein signaling molecule, for example a Hepatitis C virus (HCV) core protein, the sensor molecule, for example (SEQ ID NO. 28, SEQ ID NO. 29) adopts a conformation that cleaves a tagged reporter molecule, for example *in trans* (SEQ ID NO. 22) providing cleavage of the reporter molecule at 5'-NYG/N-3', where / represents the cleavage site, N represents any nucleotide, Y represents any pyrimidine nucleotide, and G represents Guanosine. Alternatively, a sensor molecule/reporter molecule complex provides cleavage of a tagged reporter molecule *in cis*, resulting in the release of the Tag, for example Tag-NNNN. The Tag can comprise beacons, small molecules, fluorophores, chemophores, ionophores, radio-isotopes, photophores, peptides, proteins, enzymes, antibodies, nucleic acids, and enzymatic nucleic acids.

Figure 21 shows a non-limiting example of the attachment of a high turnover protein enzyme, for example luciferase, horseradish peroxidase, beta-galactosidase, or alkaline phosphatase as a reporter molecule of a nucleic acid sensor molecule of the invention, for example a Zinzyme sensor molecule. The coupling chemistry used to attach the enzyme to the 3'-end of an oligonucleotide comprises oxidation of a *cis* diol (that can be present in a nucleotide or non-nucleotide moiety, for example an abasic derivative) followed by conjugation of a free amine of the protein or linker conjugated to the protein and reduction with sodium borohydride. Alternately, R is a phosphoramidite moiety, wherein a protein enzyme conjugated nucleoside or abasic moiety is coupled to the 5'-end of an oligonucleotide.

Figure 22 shows a non-limiting example of the use of a high turnover protein enzyme, for example luciferase, horseradish peroxidase, beta-galactosidase, or alkaline phosphatase, used as a component of a reporter molecule in conjunction with a nucleic acid sensor molecule. A system comprising a solution phase and a solid phase is used, wherein a biotin conjugated Zinzyme sensor molecule is used to detect the presence of a target signaling molecule (for example SEQ ID NO 31). In the presence of a target signaling molecule ("target" in the figure), the reporter molecule component of the sensor molecule is released from the sensor molecule when the sensor molecule interacts with the target signaling molecule in solution. The solution phase components are passed through a solid phase derivatized with avidin, streptavidin, or neutravidin. The eluent is assayed to indicate the presence of the high turnover enzyme by



providing substrate for the enzyme. Enzyme activity is indicative of the presence of the target signaling molecule in the system. Alternatively, the sensor molecule is attached to a solid support, for example covalently, wherein a sample is passed through or is passed over the support bound sensor molecule. The eluent is assayed to indicate the presence of the high turnover enzyme by providing substrate for the enzyme. Enzyme activity is indicative of the presence of the target signaling molecule in the system.

Figure 23 shows a non-limiting example of a ligase sensor molecule with an enzymatic reporter component. The system shown comprises a sensor molecule covalently attached to a surface and a separate reporter molecule, for example a high turnover protein enzyme reporter molecule which can be attached to a nucleic acid molecule, or is optionally not attached to a nucleic acid molecule. Other reporter molecules can be used in the system, including but not limited to beacons, small molecules, fluorophores, chemophores, ionophores, radio-isotopes, photophores, peptides, proteins, antibodies, nucleic acids, and enzymatic nucleic acids. In the presence of a target signaling molecule ("target" in the figure), the nucleic acid sensor molecule interacts with the target signaling molecule under conditions suitable for the ligation of the reporter molecule to the nucleic acid sensor molecule. The surface is washed to remove any unbound reporter molecules and is subsequently assayed for the presence of the reporter molecule, for example by providing a substrate for a high turnover protein enzyme reporter molecule and measuring conversion of the substrate.

Figure 24 shows a non-limiting example of a scheme for selecting and utilizing a ligase sensor molecule of the invention such as the construct described in Figure 23. The sensor molecule is selected from a motif comprising three regions including a 5'-constant region that is used as a template for polymerase activity, a variable region (for example 50 nucleotides), and a 3'-constant region that comprises the target signaling molecule to be detected by the sensor molecule. A restriction enzyme cleavage site is introduced between the 3'-constant region and the variable region of the construct. Furthermore, a biotin conjugated reporter molecule, for example selected from the group comprising beacons, small molecules, fluorophores, chemophores, ionophores, radio-isotopes, photophores, peptides, proteins, antibodies, nucleic acids, and enzymatic nucleic acids, is used to isolate sensor molecules that are selected for the capability to ligate the reporter molecule. Once sensor molecules are identified that can ligate the reporter molecule, the 3'-constant region representing the target signaling molecule is cleaved from the construct. In a system, for example one in which the nucleic acid sensor molecule is attached to a surface, interaction of the target signaling molecule with the nucleic acid sensor molecule under conditions suitable for the ligation of the reporter molecule to the nucleic acid sensor

molecule. The surface is washed to remove any unbound reporter molecules and is subsequently assayed for the presence of the reporter molecule, for example by providing a substrate for a high turnover protein enzyme reporter molecule and measuring conversion of the substrate.

Figure 25 shows a non-limiting example of a process whereby a nucleic acid signaling molecule is used in a nucleic acid circuit. The nucleic acid sensor molecule shown in the figure can be used to open or close an electronic circuit. In response to a target signaling agent, for example current, the nucleic acid sensor molecule catalyzes a chemical reaction comprising ligation in response to a predetermined current or cleavage in response to a predetermined current. The nucleic acid circuit is thereby modulated between an open and a closed state based on the predetermined input current that is applied to the circuit. A plurality of such circuits that comprise nucleic acid sensor modulation can be used in a variety of electronic devices, and can substitute solid state or silicon-based circuits in such devices. For example, computer processors comprising a plurality of nucleic acid sensor molecule based-circuits can be used in a computer device. Open and closed nucleic acid sensor molecule based-circuits can be used to generate or respond to binary code, creating a readable output.

Figure 26 shows a non limiting example of target signaling molecule inactivation of a zinzyme sensor molecule. In the absence of the target (SEQ ID NO. 31), the zinzyme sensor molecule (SEQ ID NO. 32) catalyzes the cleavage of a reporter molecule (SEQ ID NO. 33).

Figure 27 shows a non-limiting example of target signaling molecule activation of a zinzyme sensor molecule. In the presence of the target (SEQ ID NO. 34), the zinzyme sensor molecule (SEQ ID NO. 35) catalyzes the cleavage of a reporter molecule (SEQ ID NO. 36).

Figure 28 shows a non-limiting example of a nucleic acid sensor molecule that is modulated by a protein target signaling molecule, Erk. In the presence of the target protein (Erk), the nucleic acid sensor molecule (SEQ ID NO. 39) catalyzes the cleavage of a reporter molecule.

Figure 29 shows a non-limiting example of a "half-zinzyme" nucleic acid sensor molecule that is modulated by the 5'-UTR of the Hepatitis C virus (HCV 5'-UTR). The figure shows both inactive and active forms of the zinzyme sensor molecule (SEQ ID NO. 42). In the presence of the target signaling oligonucleotide (SEQ ID NO. 43) which represents the stem loop IIIB of the HCV 5'-UTR, the zinzyme sensor demonstrates an activity increase of three logs in cleaving the reporter molecule component of the sensor molecule as shown in the graph (+ oligo target) as compared to the sensor molecule in the absence of the target. In the presence of the full length 350 nt. HCV 5'-UTR, the zinzyme sensor molecule demonstrates an almost one log increase in activity in cleaving the reporter molecule component of the sensor molecule.

#### Detection of Target Signaling Molecules

In one embodiment, the invention features several approaches to detecting signaling agents, ligands and/or target signaling molecules in a system using nucleic acid molecules. In all cases, activity of the nucleic acid is modulated via interaction of the nucleic acid with the target signaling agent, ligand and/or target signaling molecule.

In one embodiment, the present invention utilizes at least three oligonucleotide sequences for proper function: nucleic acid sensor molecule, reporter molecule, and target signaling molecule. The nucleic acid sensor molecule is comprised of a sensor component, enzymatic nucleic acid component, and a linker between them which can be present or absent. The nucleic acid sensor molecule (**Figure 6**), is in its inactive state when the sensor component binds to the nucleic acid sensor molecule in the enzymatic nucleic acid component. The sensor component can bind to the substrate binding regions or nucleotides that contribute to the secondary or tertiary structure of the enzymatic nucleic acid component. For example, the sensor component can bind to nucleotides located within the nucleic acid sensor molecule, which can disrupt catalytic activity. The reporter molecule can be able to bind to the nucleic acid sensor molecule, but a catalytic activity would be inhibited since the molecule is structurally inactive. Alternatively, the sensor component can bind to the substrate binding region(s) of the enzymatic nucleic acid component, which can prevent the reporter molecule from binding to the nucleic acid sensor molecule. The sensor component cannot be cleaved because the cleavage site would contain either a chemical modification which prevents cleavage or an inappropriate sequence. For example, hammerhead ribozymes need to have a NUH motif in the molecule to be cleaved (H is adenosine, cytidine, or uridine) for proper cleavage. By adding a guanosine at the H position in the RNA to be cleaved, cleavage can be inhibited.

In the presence of the target signaling molecule, the sensor component can disassociate from the enzymatic nucleic acid component and bind to the target signaling molecule preferentially. The sensor component can preferentially bind to the target signaling molecule which results in the formation of a more stable complex. For example, the sensor component can bind to more nucleotides on the target signaling molecule than on the nucleic acid sensor molecule. Binding to a larger number of nucleotides can have increased chemical stability and therefore is preferred over binding to a smaller number of nucleotides.

When the sensor component is bound to the target signaling molecule and the reporter molecule binds to the nucleic acid sensor molecule, a reaction can be catalyzed on the reporter molecule by the enzymatic nucleic acid component. For example, the reporter molecule can be cleaved. The cleavage event can then be detected by using a number of assays. For example, electrophoresis on a polyacrylamide gel would detect not only the full length reporter

oligonucleotide but also any cleavage products that were created by the functional nucleic acid sensor molecule. The detection of these cleavage products indicate the presence of the target signaling molecule. In addition, the reporter molecule can contain a fluorescent molecule at one end which fluorescence signal is quenched by another molecule attached at the other end of the reporter molecule. Cleavage of the reporter molecule in this case results in the disassociation of the fluorescent molecule and the quench molecule, resulting in a signal. This signal can be detected and/or quantified by methods known in the art (for example see Nathan *et al.*, US Patent No. 5,871,914, Birkenmeyer, US Patent No. 5,427,930, and Lizardi *et al.*, US Patent No. 5,652,107, George *et al.*, US Patent Nos. 5,834,186 and 5,741,679, and Shih *et al.*, US Patent No. 5,589,332).

Alternatively, the sensor of the signaling molecule can comprise a separate oligonucleotide sequence, as shown for example in Figure 11, system M.

#### Target sites

Targets for useful nucleic acid sensor molecules can be determined as disclosed in Draper *et al.*, WO 93/23569; Sullivan *et al.*, WO 93/23057; Thompson *et al.*, WO 94/02595; Draper *et al.*, WO 95/04818; McSwiggen *et al.*, US Patent No. 5,525,468 and hereby incorporated by reference herein in totality. Rather than repeat the guidance provided in those documents here, below are provided specific examples of such methods, not limiting to those in the art. Nucleic acid sensor molecules to such targets are designed as described in those applications and synthesized to be tested *in vitro* and *in vivo*, as also described. Such nucleic acid sensor molecules can also be optimized and delivered as described therein.

Hammerhead, hairpin, Inozyme, Zinzyme, Amberzyme and DNAzyme-based nucleic acid sensor molecules are designed that can bind and are individually analyzed by computer folding (Jaeger *et al.*, 1989 *Proc. Natl. Acad. Sci. USA*, 86, 7706; Denman, 1993, *Biotechniques*, 15, 1090) to assess whether the nucleic acid sensor molecule sequences fold into the appropriate secondary structure. Those nucleic acid sensor molecules with unfavorable intramolecular interactions between the binding arms and the catalytic core are eliminated from consideration. Varying binding arm lengths can be chosen to optimize activity. Generally, at least 5 bases on each arm are able to bind to, or otherwise interact with, the target RNA. Nucleic acid molecules of the differing motifs are designed to anneal to various sites in the mRNA message. The binding arms are complementary to the target site sequences described above.

Hammerhead, DNAzyme, NCH, amberzyme, zinzyme or G-Cleaver-based nucleic acid sensor molecule cleavage sites were identified and were designed to anneal to various sites in the

RNA target. The binding arms are complementary to the target site sequences described above. The nucleic acid molecules were chemically synthesized. The method of synthesis used follows the procedure for normal DNA/RNA synthesis as described below and in Usman *et al.*, 1987 *J. Am. Chem. Soc.*, 109, 7845; Scaringe *et al.*, 1990 *Nucleic Acids Res.*, 18, 5433; Wincott *et al.*, 1995 *Nucleic Acids Res.* 23, 2677-2684; and Caruthers *et al.*, 1992, *Methods in Enzymology* 211,3-19.

#### Nucleic acid molecule Synthesis

The nucleic acid molecules of the invention, including certain nucleic acid sensor molecules, can be synthesized using the methods described in Usman *et al.*, 1987, *J. Am. Chem. Soc.*, 109, 7845; Scaringe *et al.*, 1990, *Nucleic Acids Res.*, 18, 5433; and Wincott *et al.*, 1995, *Nucleic Acids Res.* 23, 2677-2684 Wincott *et al.*, 1997, *Methods Mol. Bio.*, 74, 59. Such methods make use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. In a non-limiting example, small scale syntheses are conducted on a 394 Applied Biosystems, Inc. synthesizer using a 0.2  $\mu$ mol scale protocol with a 7.5 min coupling step for alkylsilyl protected nucleotides and a 2.5 min coupling step for 2'-O-methylated nucleotides. Table II outlines the amounts and the contact times of the reagents used in the synthesis cycle. Alternatively, syntheses at the 0.2  $\mu$ mol scale can be done on a 96-well plate synthesizer, such as the PG2100 instrument produced by Protogene (Palo Alto, CA) with minimal modification to the cycle. A 33-fold excess (60  $\mu$ L of 0.11 M = 6.6  $\mu$ mol) of 2'-O-methyl phosphoramidite and a 75-fold excess of S-ethyl tetrazole (60  $\mu$ L of 0.25 M = 15  $\mu$ mol) can be used in each coupling cycle of 2'-O-methyl residues relative to polymer-bound 5'-hydroxyl. A 66-fold excess (120  $\mu$ L of 0.11 M = 13.2  $\mu$ mol) of alkylsilyl (ribo) protected phosphoramidite and a 150-fold excess of S-ethyl tetrazole (120  $\mu$ L of 0.25 M = 30  $\mu$ mol) can be used in each coupling cycle of ribo residues relative to polymer-bound 5'-hydroxyl. Average coupling yields on the 394 Applied Biosystems, Inc. synthesizer, determined by colorimetric quantitation of the trityl fractions, are typically 97.5-99%. Other oligonucleotide synthesis reagents for the 394 Applied Biosystems, Inc. synthesizer include; detritylation solution is 3% TCA in methylene chloride (ABI); capping is performed with 16% *N*-methyl imidazole in THF (ABI) and 10% acetic anhydride/10% 2,6-lutidine in THF (ABI); oxidation solution is 16.9 mM I<sub>2</sub>, 49 mM pyridine, 9% water in THF (PERSEPTIVE™). Burdick & Jackson Synthesis Grade acetonitrile is used directly from the reagent bottle. S-Ethyltetrazole solution (0.25 M in acetonitrile) is made up from the solid obtained from American International Chemical, Inc. Alternately, for the introduction of phosphorothioate linkages, Beaucage reagent (3H-1,2-Benzodithiol-3-one 1,1-dioxide 0.05 M in acetonitrile) is used.

Cleavage from the solid support and deprotection of the oligonucleotide is typically performed using either a two-pot or one-pot protocol. For the two-pot protocol, the polymer-bound trityl-on oligoribonucleotide is transferred to a 4 mL glass screw top vial and suspended in a solution of 40% aq. methylamine (1 mL) at 65 °C for 10 min. After cooling to -20 °C, the supernatant is removed from the polymer support. The support is washed three times with 1.0 mL of EtOH:MeCN:H<sub>2</sub>O/3:1:1, vortexed and the supernatant is then added to the first supernatant. The combined supernatants, containing the oligoribonucleotide, are dried to a white powder. The base deprotected oligoribonucleotide is resuspended in anhydrous TEA/HF/NMP solution (300 µL of a solution of 1.5 mL N-methylpyrrolidinone, 750 µL TEA and 1 mL TEA•3HF to provide a 1.4 M HF concentration) and heated to 65 °C. After 1.5 h, the oligomer is quenched with 1.5 M NH<sub>4</sub>HCO<sub>3</sub>.

Alternatively, for the one-pot protocol, the polymer-bound trityl-on oligoribonucleotide is transferred to a 4 mL glass screw top vial and suspended in a solution of 33% ethanolic methylamine/DMSO: 1/1 (0.8 mL) at 65°C for 15 min. The vial is brought to r.t. TEA•3HF (0.1 mL) is added and the vial is heated at 65 °C for 15 min. The sample is cooled at -20 °C and then quenched with 1.5 M NH<sub>4</sub>HCO<sub>3</sub>. An alternative deprotection cocktail for use in the one pot protocol comprises the use of aqueous methylamine (0.5 ml) at 65°C for 15 min followed by DMSO (0.8 ml) and TEA•3HF (0.3 ml) at 65°C for 15 min. A similar methodology can be employed with 96-well plate synthesis formats by using a Robbins Scientific Flex Chem block, in which the reagents are added for cleavage and deprotection of the oligonucleotide.

For anion exchange desalting of the deprotected oligomer, the TEAB solution is loaded onto a Qiagen 500<sup>®</sup> anion exchange cartridge (Qiagen Inc.) that is prewashed with 50 mM TEAB (10 mL). After washing the loaded cartridge with 50 mM TEAB (10 mL), the RNA is eluted with 2 M TEAB (10 mL) and dried down to a white powder.

For purification of the trityl-on oligomers, the quenched NH<sub>4</sub>HCO<sub>3</sub> solution is loaded onto a C-18 containing cartridge that had been prewashed with acetonitrile followed by 50 mM TEAA. After washing the loaded cartridge with water, the RNA is detritylated with 0.5% TFA for 13 min. The cartridge is then washed again with water, salt exchanged with 1 M NaCl and washed with water again. The oligonucleotide is then eluted with 30% acetonitrile. Alternatively, for oligonucleotides synthesized in a 96-well format, the crude trityl-on oligonucleotide is purified using a 96-well solid phase extraction block packed with C18 material, on a Bahdan Automation workstation.

The average stepwise coupling yields are typically >98% (Wincott *et al.*, 1995 *Nucleic Acids Res.* 23, 2677-2684). Those of ordinary skill in the art will recognize that the scale of synthesis can be adapted as larger or smaller than the example described above including but not limited to 96 well format, all that is important is the ratio of chemicals used in the reaction.

To ensure the quality of synthesis of nucleic acid molecules of the invention, quality control measures are utilized for the analysis of nucleic acid material. Capillary Gel Electrophoresis, for example using a Beckman MDQ CGE instrument, can be utilized for rapid analysis of nucleic acid molecules, by introducing sample on the short end of the capillary. In addition, mass spectrometry, for example using a PE Biosystems Voyager-DE MALDI instrument, in combination with the Bohdan workstation, can be utilized in the analysis of oligonucleotides, including oligonucleotides synthesized in the 96-well format.

The nucleic acids of the invention can also be synthesized in two parts and annealed to reconstruct the nucleic acid sensor molecules (Chowrira and Burke, 1992 *Nucleic Acids Res.*, 20, 2835-2840). The nucleic acids are also synthesized enzymatically using a variety of methods known in the art, for example as described in Havlina, International PCT publication No. WO 9967413, or from DNA templates using bacteriophage T7 RNA polymerase (Milligan and Uhlenbeck, 1989, *Methods Enzymol.* 180, 51). Other methods of enzymatic synthesis of the nucleic acid molecules of the invention are generally described in Kim *et al.*, 1995, *Biotechniques*, 18, 992; Hoffman *et al.*, 1994, *Biotechniques*, 17, 372; Cazenare *et al.*, 1994, *PNAS USA*, 91, 6972; Hyman, US Patent No. 5,436,143; and Karpeisky *et al.*, International PCT publication No. WO 98/28317)

Alternatively, the nucleic acid molecules of the present invention can be synthesized separately and joined together post-synthetically, for example by ligation (Moore *et al.*, 1992, *Science* 256, 9923; Draper *et al.*, International PCT publication No. WO 93/23569; Shabarova *et al.*, 1991, *Nucleic Acids Research* 19, 4247; Bellon *et al.*, 1997, *Nucleosides & Nucleotides*, 16, 951; Bellon *et al.*, 1997, *Bioconjugate Chem.* 8, 204).

The nucleic acid molecules of the present invention are preferably modified to enhance stability by modification with nuclease resistant groups, for example, 2'-amino, 2'-C-allyl, 2'-fluoro, 2'-O-methyl, 2'-H (for a review see Usman and Cedergren, 1992, *TIBS* 17, 34; Usman *et al.*, 1994, *Nucleic Acids Symp. Ser.* 31, 163). Nucleic acid sensor molecules are purified by gel electrophoresis using known methods or are purified by high pressure liquid chromatography (HPLC; See Wincott *et al.*, *Supra*, the totality of which is hereby incorporated herein by reference) and are re-suspended in water.

The sequences of the nucleic acids that are chemically synthesized, useful in this study, are shown in Table III. Those in the art will recognize that these sequences are representative only of many more such sequences where the enzymatic portion of the nucleic acid (all but the binding arms) is altered to affect activity. The nucleic acid construct sequences listed in Table III can be formed of ribonucleotides or other nucleotides or non-nucleotides. Such nucleic acids with enzymatic activity are equivalent to the nucleic acids described specifically in the Table.

#### Optimizing nucleic acid molecule activity

Synthesizing nucleic acid molecules with modifications (base, sugar and/or phosphate) that prevent their degradation by serum ribonucleases can increase their potency (see *e.g.*, Eckstein *et al.*, International Publication No. WO92/07065; Perrault *et al.*, 1990 *Nature* 344, 565; Pieken *et al.*, 1991, *Science* 253, 314; Usman and Cedergren, 1992, *Trends in Biochem. Sci.* 17, 334; Usman *et al.*, International Publication No. WO93/15187; Rossi *et al.*, International Publication No. WO 91/03162; Sproat, US Patent No. 5,334,711; and Burgin *et al.*, *supra*; all of these describe various chemical modifications that can be made to the base, phosphate and/or sugar moieties of the nucleic acid molecules described herein. All these references are incorporated by reference herein. Modifications which enhance their efficacy in cells, and removal of bases from nucleic acid molecules to shorten oligonucleotide synthesis times and reduce chemical requirements are preferably desired.

There are several examples in the art describing sugar, base and phosphate modifications that can be introduced into nucleic acid molecules with significant enhancement in their nuclease stability and efficacy. For example, oligonucleotides are modified to enhance stability and/or enhance biological activity by modification with nuclease resistant groups, for example, 2'-amino, 2'-C-allyl, 2'-fluoro, 2'-O-methyl, 2'-H, nucleotide base modifications (for a review see Usman and Cedergren, 1992, *TIBS* 17, 34; Usman *et al.*, 1994, *Nucleic Acids Symp. Ser.* 31, 163; Burgin *et al.*, 1996, *Biochemistry*, 35, 14090). Sugar modifications of nucleic acid molecules have been extensively described in the art (see Eckstein *et al.*, International Publication PCT No. WO 92/07065; Perrault *et al.* *Nature*, 1990, 344, 565-568; Pieken *et al.* *Science*, 1991, 253, 314-317; Usman and Cedergren, *Trends in Biochem. Sci.*, 1992, 17, 334-339; Usman *et al.* International Publication PCT No. WO 93/15187; Sproat, US Patent No. 5,334,711 and Beigelman *et al.*, 1995, *J. Biol. Chem.*, 270, 25702; Beigelman *et al.*, International PCT publication No. WO 97/26270; Beigelman *et al.*, US Patent No. 5,716,824; Usman *et al.*, US patent No. 5,627,053; Woolf *et al.*, International PCT Publication No. WO 98/13526; Thompson *et al.*, USSN 60/082,404 which was filed on April 20, 1998; Karpeisky *et al.*, 1998,



*Tetrahedron Lett.*, 39, 1131; Earnshaw and Gait, 1998, *Biopolymers (Nucleic acid Sciences)*, 48, 39-55; Verma and Eckstein, 1998, *Annu. Rev. Biochem.*, 67, 99-134; and Burlina *et al.*, 1997, *Bioorg. Med. Chem.*, 5, 1999-2010; all of the references are hereby incorporated by reference herein in their totalities). Such publications describe general methods and strategies to determine the location of incorporation of sugar, base and/or phosphate modifications and the like into nucleic acid sensor molecule molecules without inhibiting catalysis. In view of such teachings, similar modifications can be used as described herein to modify the nucleic acid molecules of the instant invention.

While chemical modification of oligonucleotide internucleotide linkages with phosphorothioate, phosphorothioate, and/or 5'-methylphosphonate linkages improves stability, many of these modifications can cause some toxicity. Therefore when designing nucleic acid molecules the amount of these internucleotide linkages should be minimized. The reduction in the concentration of these linkages should lower toxicity resulting in increased efficacy and higher specificity of these molecules.

Nucleic acid molecules having chemical modifications which maintain or enhance activity are provided. Such nucleic acid is also generally more resistant to nucleases than unmodified nucleic acid. Thus, in the presence of biological fluids, or in cells, the activity can not be significantly lowered. Clearly, nucleic acid molecules must be resistant to nucleases in order to function as effective diagnostic agents, whether utilized *in vitro* and/or *in vivo*. Improvements in the synthesis of RNA and DNA (Wincott *et al.*, 1995 *Nucleic Acids Res.* 23, 2677; Caruthers *et al.*, 1992, *Methods in Enzymology* 211,3-19; Karpeisky *et al.*, International PCT publication No. WO 98/28317) (incorporated by reference herein) have expanded the ability to modify nucleic acid molecules by introducing nucleotide modifications to enhance their nuclease stability as described above.

In another aspect the nucleic acid molecules comprise a 5' and/or a 3' - cap structure.

In one embodiment, the invention features modified nucleic acid molecules with phosphate backbone modifications comprising one or more phosphorothioate, phosphorodithioate, methylphosphonate, morpholino, amidate carbamate, carboxymethyl, acetamidate, polyamide, sulfonate, sulfonamide, sulfamate, formacetal, thioformacetal, and/or alkylsilyl, substitutions. For a review of oligonucleotide backbone modifications see Hunziker and Leumann, 1995, *Nucleic Acid Analogues: Synthesis and Properties*, in *Modern Synthetic Methods*, VCH, 331-417, and Mesmaeker *et al.*, 1994, *Novel Backbone Replacements for Oligonucleotides*, in *Carbohydrate Modifications in Antisense Research*, ACS, 24-39. These references are hereby incorporated by reference herein.

In connection with 2'-modified nucleotides as described for the present invention, by "amino" is meant 2'-NH<sub>2</sub> or 2'-O- NH<sub>2</sub>, which can be modified or unmodified. Such modified groups are described, for example, in Eckstein *et al.*, U.S. Patent 5,672,695 and Karpeisky *et al.*, WO 98/28317, respectively, which are both incorporated by reference herein in their entireties.

Various modifications to nucleic acid (*e.g.*, nucleic acid sensor molecule) structure can be made to enhance the utility of these molecules. Such modifications enhance shelf-life, half-life *in vitro*, stability, and ease of introduction of such oligonucleotides to the target site, *e.g.*, to enhance penetration of cellular membranes, and confer the ability to recognize and bind to targeted cells.

### Examples

The following are non-limiting examples showing techniques useful in isolating nucleic acid molecules of the instant invention.

#### Example 1: Diagnostic screen

A series of sensor molecules with trans-acting sensor component sequences were designed. Table III shows the sequences that were used in this test. Sequences with names beginning with R- were the reporter sequences used in this experiment, and those beginning with SM- were nucleic acid sensor molecules. Sequences beginning with S- were sensor component sequences that were designed to bind to portions of the sensor molecule sequences (to varying degrees) and to prevent the sensor molecule from binding and cleaving reporter molecules; these sequences are shown in lower case because they were synthesized using 2'-O-methyl nucleotides in order to increase binding affinity. The one sequence labeled T-2a represents the target signaling molecule sequence which was designed to bind to the sensor component sequences so as to prevent them from inhibiting the sensor molecule activity. The system construct is shown in Figure 15.

Figure 16 shows the results of testing some of these sensor molecule/sensor component combinations in a cleavage assay. The reporter molecules were 5'-end labeled with <sup>32</sup>P-phosphate and incubated for 12 or 60 minutes in either: (1) buffer alone (50 mM Tris, pH 7.5, 10 mM MgCl<sub>2</sub>), or in the presence of (2) 10 nM sensor molecule, (3) 10 nM sensor molecule plus 20 nM sensor component, (4) 10 nM sensor molecule plus 200 nM sensor component, or (5) 10 nM sensor molecule plus 20 nM sensor component and 500 nM target signaling molecule. At the end of the incubation the reactions were loaded onto a PAGE gel to separate cleaved reporter from uncleaved reporter. The gel was imaged on a Molecular Dynamics phosphorimager and quantitated to determine the percent of reporter molecule cleaved under each set of conditions.

Control reactions were carried out to ensure that addition of sensor component or target signaling sequence, without sensor molecule, did not result in reporter cleavage; only 0.2-0.4% of reporter was cleaved under these conditions.

Figure 16 shows that sensor molecule alone results in 40-60% cleavage of the reporter molecule after 1 minute, and 85% cleavage after 60 minutes for three sensor molecules. When 20 nM sensor component is added to the reaction, the cleavage activity is reduced by 30-70%. When 200 nM sensor component is added, the cleavage activity is reduced by 50-99%. Finally, addition of 500 nM target signaling molecule to a reaction containing 10 nM sensor molecule and 20 nM target signaling molecule results in almost complete recovery of the cleavage activity up to the level observed with sensor molecule alone.

#### Example 2: Auto-ligating Nucleic Acid Molecules:

Figure 17 is a schematic representation of the method of the invention used to isolate nucleic acid molecules capable of auto-ligation reactions useful, for example, in diagnostic applications. Figure 17a shows the general selection scheme used for isolating active sequences. A random pool of nucleic acid, such as RNA is combined with a substrate molecule comprising the structure R1-O-R2-Biotin, wherein R1 is selected from the group consisting of methyl, hydrogen, phosphate, nucleoside, nucleotide, oligonucleotide, R2 is selected from the group comprising molecules capable of generating a detectable signal, such as molecular beacons, small molecules, fluorophores, chemophores, ionophores, radio-isotopes, photophores, peptides, proteins, enzymes, antibodies, nucleic acids, and enzymatic nucleic acids, L represents a linker which can be present or absent, and "-" represents a covalent bond. Catalytically active sequences are biotinylated. The reaction mixture is passed over a solid support derivatized with Avidin, resulting in the capture of the biotinylated, catalytically active sequence pool. The support bound sequences are amplified by methods known in the art. Figure 17b shows the selection of the initial pool of sequences that provide ligation activity, and subsequent selection of molecules that are active in the presence of a ligand. Initially, selection of catalytic sequences takes place in the absence of the ligand. The active molecules isolated from the first round of selection that initially bind to the Avidin derivatized support are eliminated. Molecules that pass through the support are re-selected in the presence of the ligand. The re-selected pool that binds to the support after reaction in the presence of the ligand is amplified by methods known in the art and transcribed for subsequent rounds of selection. Figure 17c shows another selection strategy for isolating nucleic acid molecules capable of autoligation in the presence of a ligand.

In this case, an initial selection takes place in the absence of the ligand to select sequences with autoligation activity. This pool is mutagenized by methods known in the art. The resulting mutagenized pool is selected for ligand binding activity by methods known in the art, for example, by using ligand affinity chromatography or gel shift assays. The resulting pool is mutagenized by methods known in the art. The original selection (for activity) is repeated in the presence of the ligand of diagnostic interest, with counterselection for molecules that react in the absence of the ligand.

Example 3: Isomerase Nucleic Acid Molecules:

Figure 18 is a schematic representation of the method of the invention used to isolate nucleic acid sensor molecules capable of catalyzing isomerization reactions useful, for example in diagnostic applications. R1 and R2 represent compounds, which can be the same or different, capable of generating a detectable signal or quenching a detectable signal when an isomerization event takes place, comprising molecular beacons, small molecules, fluorophores, chemophores, ionophores, radio-isotopes, photophores, peptides, proteins, enzymes, antibodies, nucleic acids, and enzymatic nucleic acids, L represents a linker which can be present or absent, and “-” represents a covalent bond. Figure 18a shows the general selection scheme used for isolating active sequences. A random pool of nucleic acid sequences are passed over the complex of interest, derivatized to a solid support. The representative example of the complex shown in the figure consists of two fluorescent molecules joined together via a cis-carbon double bond linkage. Alternatively, a trans-carbon double bond linkage can be used. The selection pool is enriched and mutagenized throughout multiple generations to generate a diverse pool of “cis” binding sequences. Cis-binding nucleic acid molecules are then loaded onto the resin and the corresponding trans isomer of the complex is used to elute sequences that bind the trans-isomer tighter than the cis-isomer. Figure 18b shows how the concentration of cis-isomer on the resin and the concentration of trans-isomer eluant can be manipulated in order to select sequences that prefer binding to one isomer over the other, and can therefor drive the reaction in the desired direction. Figure 18c shows a selection scheme for isolating ligand dependent nucleic acid isomerase molecules from the initial selection pool from Figure 18a. A counter-selection takes place in which sequences that are bound to the cis-isomer complex are eluted with the ligand of diagnostic interest. An additional counter-selection takes place in which sequences that are bound to the cis-isomer complex are eluted with the ligand of diagnostic interest. A selection then takes place in which sequences remaining from the counter-selection rounds that are bound to the cis-isomer complex are eluted with a mixture of the ligand of diagnostic interest and the

trans-isomer complex, the eluted ligand dependent nucleic acid catalyst sequences are amplified and transcribed by methods known in the art.

Example 4: Detection of HCV RNA:

A nucleic acid sensor molecule of the instant invention can be utilized to detect the presence of hepatitis C virus (HCV) in a sample of human blood. A system comprising a human blood sample, a reporter molecule such as a high turnover enzyme, and a nucleic acid sensor molecule attached to a solid support surface is used. The nucleic acid sensor molecule comprises an enzymatic nucleic acid component including an HCV specific sensor component, wherein in response to an interaction of HCV RNA or HCV core proteins with the nucleic acid sensor molecule, the enzymatic nucleic acid component catalyzes a chemical reaction. The reaction can comprise cleavage and release of a reporter molecule when HCV RNA is used as a target signaling agent (see for example Figure 19), or when HCV core proteins are used as a target signaling agent (see for example Figure 20). Alternatively, the reaction can comprise the attachment of the reporter molecule to the nucleic acid sensor molecule in the presence of the HCV target (see for example Figure 23 or Figure 24). In the case of a sensor molecule that ligates a reporter molecule, the system is subjected to conditions under which free reporter molecules are removed from the system, for example, by washing the surface of the solid support.

The reporter molecule in the system can comprise a conjugated enzyme, such as luciferase, alkaline phosphatase, or horseradish peroxidase. Covalent attachment of the reporter molecule to the nucleic acid sensor molecule takes place in the presence of HCV RNA or core protein. The system is subjected to conditions that cause free reporter molecule to be removed from the system, for example, washing the surface of a solid phase system. A substrate for the conjugated enzyme is contacted with the system under conditions where conversion of the substrate by the immobilized enzyme generates an amplified signal, for example a precipitate, that is detected on the surface of the system (see Figure 23 or Figure 24).

A system in which cleavage of a reporter molecule rather than ligation is used to detect the presence of a target signaling molecule is shown in Figure 22. An example for the attachment of a reporter enzyme to a nucleic acid sequence is shown in Figure 21. A system comprising a solution phase and a solid phase is used, wherein a biotin conjugated Zinzyme sensor molecule is used to detect the presence of a target signaling molecule (for example HCV RNA). In the presence of the HCV RNA target signaling molecule ("target" in the figure), the reporter molecule component of the sensor molecule is released from the sensor molecule when

the sensor molecule interacts with the target signaling molecule in solution. The solution phase components are passed through a solid phase derivatized with avidin, streptavidin, or neutravidin. The eluent is assayed to indicate the presence of the high turnover enzyme by providing substrate for the enzyme. Enzyme activity is indicative of the presence of the HCV RNA in the system. Alternatively, the sensor molecule is attached to a solid support, for example covalently, wherein a sample is passed through or is passed over the support bound sensor molecule. The eluent is assayed to indicate the presence of the high turnover enzyme by providing substrate for the enzyme. Enzyme activity is indicative of the presence of the HCV RNA in the system.

The use of nucleic acid sensor molecules as described herein is amenable to point of care applications, enabling the simple and efficient detection of analytes in a clinical setting.

Example 5: Nucleic acid sensor circuit

Figure 25 describes a process whereby a nucleic acid signaling molecule is used in a nucleic acid circuit. The nucleic acid sensor molecule can be used to open or close an electronic circuit. In response to a target signaling agent, for example current, the nucleic acid sensor molecule catalyzes a chemical reaction comprising ligation in response to a predetermined current or cleavage in response to a predetermined current. The nucleic acid circuit is thereby modulated between an open and a closed state based on the predetermined input current that is applied to the circuit. A plurality of such circuits that comprise nucleic acid sensor modulation can be used in a variety of electronic devices, and can substitute solid state or silicon-based circuits in such devices. For example, computer processors comprising a plurality of nucleic acid sensor molecule based-circuits can be used in a computer device. Open and closed nucleic acid sensor molecule based-circuits can be used to generate or respond to binary code, creating a readable output. Processing of nucleic acids by nucleic acid sensor molecules can be used to generate more complex code, for example where particular nucleic acid sequences represent different code variables.

Example 6: Target inhibition of nucleic acid sensor molecule

Figure 26 shows a non-limiting example of target signaling molecule inactivation of a zinzyme sensor molecule. In the absence of the target (SEQ ID NO. 31), the zinzyme sensor molecule (SEQ ID NO. 32) catalyzes the cleavage of a reporter molecule (SEQ ID NO. 33). Reaction conditions: 140mM KCl, 10mM NaCl, 20 mM HEPES pH 7.4, 1mM MgCl<sub>2</sub>, 1mM CaCl<sub>2</sub>, 400 nM Nucleic acid sensor, 400 nM Target, Trace of labeled reporter (~10 nM), 25μl

reaction volume, Nucleic acid sensor, target and reporter were heated at 75°C for 3 min, cooled to 37°C and cleavage initiated by the addition of MgCl<sub>2</sub> and CaCl<sub>2</sub>.

Example 6: Target activation of nucleic acid sensor molecule

Figure 27 shows a non-limiting example of target signaling molecule activation of a zinzyme sensor molecule. In the presence of the target (SEQ ID NO. 34), the zinzyme sensor molecule (SEQ ID NO. 35) catalyzes the cleavage of a reporter molecule (SEQ ID NO. 36). Reaction conditions: 140mM KCl, 10mM NaCl, 20 mM HEPES pH 7.4, 1mM MgCl<sub>2</sub>, 1mM CaCl<sub>2</sub>, 400 nM Nucleic acid sensor, 400 nM Target, Trace of labeled reporter (~10 nM), 25µl reaction volume, Nucleic acid sensor, target and reporter were heated at 75°C for 3 min, cooled to 37°C and cleavage initiated by the addition of MgCl<sub>2</sub> and CaCl<sub>2</sub>.

Example 7: Protein (Erk) target activation of nucleic acid sensor molecule

Figure 28 shows a non-limiting example of a nucleic acid sensor molecule that is modulated by a protein target signaling molecule, Erk. In the presence of the target protein (Erk), the nucleic acid sensor molecule (SEQ ID NO. 39) catalyzes the cleavage of a reporter molecule. Reaction conditions: 100mM KCl, 1mM MgCl<sub>2</sub>, 10mM Tris 7.5, 10µM ERK protein, 1µM HH ribozyme, Vf=19µl, 34°C for 30 minutes, trace 5' labeled substrate (1µl).

Example 8: Half-zinzyme nucleic acid sensor molecule

Figure 29 shows a non-limiting example of a "half-zinzyme" nucleic acid sensor molecule with a PEG linker that is modulated by the 5'-UTR of the Hepatitis C virus (HCV 5'-UTR). The figure shows both inactive and active forms of the zinzyme sensor molecule (SEQ ID NO. 42). In the presence of the target signaling oligonucleotide (SEQ ID NO. 43) which represents the stem loop IIIB of the HCV 5'-UTR, the zinzyme sensor demonstrates an activity increase of three logs in cleaving the reporter molecule component of the sensor molecule as shown in the graph (+ oligo target) as compared to the sensor molecule in the absence of the target. In the presence of the full length 350 nt. HCV 5'-UTR, the zinzyme sensor molecule demonstrates an almost one log increase in activity in cleaving the reporter molecule component of the sensor molecule. Reaction conditions: 140mM KCl, 10mM NaCl, 20 mM HEPES pH 7.4, 1mM MgCl<sub>2</sub>, 1mM CaCl<sub>2</sub>, 400 nM Nucleic acid sensor, 400 nM Target, Trace of labeled reporter (~10 nM), 25µl reaction volume, Nucleic acid sensor, target and reporter were heated at 75°C for 3 min, cooled to 37°C and cleavage initiated by the addition of MgCl<sub>2</sub> and CaCl<sub>2</sub>.

Other uses

The nucleic acid sensor molecules of this invention can be used as diagnostic tools to examine genetic drift and mutations within diseased cells or to detect the presence of a specific

RNA in a cell. The close relationship between nucleic acid sensor molecule activity and the structure of the target RNA allows the detection of mutations in any region of the molecule which alters the base-pairing and three-dimensional structure of the target RNA. By using multiple nucleic acid sensor molecules described in this invention, one can map nucleotide changes which are important to RNA structure and function *in vitro*, as well as in cells and tissues. Cleavage of target RNAs with nucleic acid sensor molecules can be used to inhibit gene expression and define the role (essentially) of specified gene products in the progression of disease. In this manner, other genetic targets can be defined as important mediators of the disease. These experiments can lead to better treatment of the disease progression by affording the possibility of combinational therapies (*e.g.*, multiple nucleic acid sensor molecules targeted to different genes, nucleic acid target molecules coupled with known small molecule inhibitors, or intermittent treatment with combinations of nucleic acid sensor molecules and/or other chemical or biological molecules). Other *in vitro* uses of nucleic acid sensor molecules of this invention comprise detection of the presence of mRNAs associated with a disease-related condition. Such RNA is detected by determining the presence of a cleavage product after treatment with an enzymatic nucleic acid molecule using standard methodology.

In a specific example, nucleic acid sensor molecules which cleave only wild-type or mutant forms of the target RNA are used for the assay. The first nucleic acid sensor molecule is used to identify wild-type RNA present in the sample and the second nucleic acid sensor molecule is used to identify mutant RNA in the sample. As reaction controls, synthetic substrates of both wild-type and mutant RNA are cleaved by both nucleic acid sensor molecules to demonstrate the relative nucleic acid sensor molecule efficiencies in the reactions and the absence of cleavage of the "non-targeted" RNA species. The cleavage products from the synthetic substrates also serve to generate size markers for the analysis of wild-type and mutant RNAs in the sample population. Thus, each analysis can require two nucleic acid sensor molecules, two substrates and one unknown sample, which are combined into six reactions. The presence of cleavage products is determined using an RNase protection assay so that full-length and cleavage fragments of each RNA can be analyzed in one lane of a polyacrylamide gel. It is not absolutely required to quantify the results to gain insight into the expression of mutant RNAs and putative risk of the desired phenotypic changes in target cells. The expression of mRNA whose protein product is implicated in the development of the phenotype is adequate to establish risk. If probes of comparable specific activity are used for both transcripts, then a qualitative comparison of RNA levels is adequate and decrease the cost of the initial diagnosis. Higher



mutant form to wild-type ratios are correlated with higher risk whether RNA levels are compared qualitatively or quantitatively.

#### Additional Uses

Potential usefulness of sequence-specific nucleic acid sensor molecules of the instant invention have many of the same applications for the study of RNA that DNA restriction endonucleases have for the study of DNA (Nathans *et al.*, 1975 *Ann. Rev. Biochem.* 44:273). For example, the pattern of restriction fragments can be used to establish sequence relationships between two related RNAs, and large RNAs can be specifically cleaved to fragments of a size more useful for study. The ability to engineer sequence specificity of the enzymatic nucleic acid molecule is ideal for cleavage of RNAs of unknown sequence. Applicant describes the use of nucleic acid molecules to detect gene expression of target genes in bacterial, microbial, fungal, viral, and eukaryotic systems including plant, or mammalian cells.

All patents and publications mentioned in the specification are indicative of the levels of skill of those skilled in the art to which the invention pertains. All references cited in this disclosure are incorporated by reference to the same extent as if each reference had been incorporated by reference in its entirety individually.

One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The methods and compositions described herein as presently representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses which are encompassed within the spirit of the invention, are defined by the scope of the claims.

It will be readily apparent to one skilled in the art that varying substitutions and modifications can be made to the invention disclosed herein without departing from the scope and spirit of the invention. Thus, such additional embodiments are within the scope of the present invention and the following claims.

The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of" and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that

various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments, optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the description and the appended claims.

In addition, where features or aspects of the invention are described in terms of Markush groups or other grouping of alternatives, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group or other group.

Other embodiments are within the following claims.

## TABLE I

### Characteristics of naturally occurring ribozymes

#### Group I Introns

- Size: ~150 to >1000 nucleotides.
- Requires a U in the target sequence immediately 5' of the cleavage site.
- Binds 4-6 nucleotides at the 5'-side of the cleavage site.
- Reaction mechanism: attack by the 3'-OH of guanosine to generate cleavage products with 3'-OH and 5'-guanosine.
- Additional protein cofactors required in some cases to help folding and maintenance of the active structure.
- Over 300 known members of this class. Found as an intervening sequence in *Tetrahymena thermophila* rRNA, fungal mitochondria, chloroplasts, phage T4, blue-green algae, and others.
- Major structural features largely established through phylogenetic comparisons, mutagenesis, and biochemical studies [i, ii].
- Complete kinetic framework established for one ribozyme [iii, iv, v, vi].
- Studies of ribozyme folding and substrate docking underway [vii, viii, ix].
- Chemical modification investigation of important residues well established [x, xi].
- The small (4-6 nt) binding site can make this ribozyme too non-specific for targeted RNA cleavage, however, the *Tetrahymena* group I intron has been used to repair a "defective" beta-galactosidase message by the ligation of new beta-galactosidase sequences onto the defective message [xii].

#### RNAse P RNA (M1 RNA)

- Size: ~290 to 400 nucleotides.
- RNA portion of a ubiquitous ribonucleoprotein enzyme.
- Cleaves tRNA precursors to form mature tRNA [xiii].
- Reaction mechanism: possible attack by  $M^{2+}$ -OH to generate cleavage products with 3'-OH and 5'-phosphate.

- RNase P is found throughout the prokaryotes and eukaryotes. The RNA subunit has been sequenced from bacteria, yeast, rodents, and primates.
- Recruitment of endogenous RNase P for therapeutic applications is possible through hybridization of an External Guide Sequence (EGS) to the target RNA [xiv, xv]
- Important phosphate and 2' OH contacts recently identified [xvi, xvii]

### Group II Introns

- Size: >1000 nucleotides.
- Trans cleavage of target RNAs recently demonstrated [xviii, xix].
- Sequence requirements not fully determined.
- Reaction mechanism: 2'-OH of an internal adenosine generates cleavage products with 3'-OH and a "lariat" RNA containing a 3'-5' and a 2'-5' branch point.
- Only natural ribozyme with demonstrated participation in DNA cleavage [xx, xxi] in addition to RNA cleavage and ligation.
- Major structural features largely established through phylogenetic comparisons [xxii].
- Important 2' OH contacts beginning to be identified [xxiii]
- Kinetic framework under development [xxiv]

### Neurospora VS RNA

- Size: ~144 nucleotides.
- Trans cleavage of hairpin target RNAs recently demonstrated [xxv].
- Sequence requirements not fully determined.
- Reaction mechanism: attack by 2'-OH 5' to the scissile bond to generate cleavage products with 2',3'-cyclic phosphate and 5'-OH ends.
- Binding sites and structural requirements not fully determined.
- Only 1 known member of this class. Found in Neurospora VS RNA.

### Hammerhead Ribozyme

(see text for references)

- Size: ~13 to 40 nucleotides.
- Requires the target sequence UH immediately 5' of the cleavage site.
- Binds a variable number nucleotides on both sides of the cleavage site.
- Reaction mechanism: attack by 2'-OH 5' to the scissile bond to generate cleavage products with 2',3'-cyclic phosphate and 5'-OH ends.
- 14 known members of this class. Found in a number of plant pathogens (virusoids) that use RNA as the infectious agent.
- Essential structural features largely defined, including 2 crystal structures [xxvi, xxvii]
- Minimal ligation activity demonstrated (for engineering through *in vitro* selection) [xxviii]
- Complete kinetic framework established for two or more ribozymes [xxix].
- Chemical modification investigation of important residues well established [xxx].

#### Hairpin Ribozyme

- Size: ~50 nucleotides.
- Requires the target sequence GUC immediately 3' of the cleavage site.
- Binds 4-6 nucleotides at the 5'-side of the cleavage site and a variable number to the 3'-side of the cleavage site.
- Reaction mechanism: attack by 2'-OH 5' to the scissile bond to generate cleavage products with 2',3'-cyclic phosphate and 5'-OH ends.
- 3 known members of this class. Found in three plant pathogen (satellite RNAs of the tobacco ringspot virus, arabis mosaic virus and chicory yellow mottle virus) which uses RNA as the infectious agent.
- Essential structural features largely defined [xxxi, xxxii, xxxiii, xxxiv]
- Ligation activity (in addition to cleavage activity) makes ribozyme amenable to engineering through *in vitro* selection [xxxv]
- Complete kinetic framework established for one ribozyme [xxxvi].

- Chemical modification investigation of important residues begun [<sup>xxxvii</sup>, <sup>xxxviii</sup>].

### Hepatitis Delta Virus (HDV) Ribozyme

- Size: ~60 nucleotides.
- Trans cleavage of target RNAs demonstrated [<sup>xxxix</sup>].
- Binding sites and structural requirements not fully determined, although no sequences 5' of cleavage site are required. Folded ribozyme contains a pseudoknot structure [<sup>xl</sup>].
- Reaction mechanism: attack by 2'-OH 5' to the scissile bond to generate cleavage products with 2',3'-cyclic phosphate and 5'-OH ends.
- Only 2 known members of this class. Found in human HDV.
- Circular form of HDV is active and shows increased nuclease stability [<sup>xli</sup>]

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Table II:

A. 2.5  $\mu$ mol Synthesis Cycle ABI 394 Instrument

Reagent	Equivalents	Amount	Wait Time* DNA	Wait Time* 2'-O-methyl	Wait Time*RNA
Phosphoramidites	6.5	163 $\mu$ L	45 sec	2.5 min	7.5 min
S-Ethyl Tetrazole	23.8	238 $\mu$ L	45 sec	2.5 min	7.5 min
Acetic Anhydride	100	233 $\mu$ L	5 sec	5 sec	5 sec
N-Methyl Imidazole	186	233 $\mu$ L	5 sec	5 sec	5 sec
TCA	176	2.3 mL	21 sec	21 sec	21 sec
Iodine	11.2	1.7 mL	45 sec	45 sec	45 sec
Beaucage	12.9	645 $\mu$ L	100 sec	300 sec	300 sec
Acetonitrile	NA	6.67 mL	NA	NA	NA

B. 0.2  $\mu$ mol Synthesis Cycle ABI 394 Instrument

Reagent	Equivalents	Amount	Wait Time* DNA	Wait Time* 2'-O-methyl	Wait Time*RNA
Phosphoramidites	15	31 $\mu$ L	45 sec	233 sec	465 sec
S-Ethyl Tetrazole	38.7	31 $\mu$ L	45 sec	233 min	465 sec
Acetic Anhydride	655	124 $\mu$ L	5 sec	5 sec	5 sec
N-Methyl Imidazole	1245	124 $\mu$ L	5 sec	5 sec	5 sec
TCA	700	732 $\mu$ L	10 sec	10 sec	10 sec
Iodine	20.6	244 $\mu$ L	15 sec	15 sec	15 sec
Beaucage	7.7	232 $\mu$ L	100 sec	300 sec	300 sec
Acetonitrile	NA	2.64 mL	NA	NA	NA

C. 0.2  $\mu$ mol Synthesis Cycle 96 well Instrument

Reagent	Equivalents:DNA/ 2'-O-methyl/Ribo	Amount: DNA/2'-O- methyl/Ribo	Wait Time* DNA	Wait Time* 2'-O- methyl	Wait Time* Ribo
Phosphoramidites	22/33/66	40/60/120 $\mu$ L	60 sec	180 sec	360sec
S-Ethyl Tetrazole	70/105/210	40/60/120 $\mu$ L	60 sec	180 min	360 sec
Acetic Anhydride	265/265/265	50/50/50 $\mu$ L	10 sec	10 sec	10 sec
N-Methyl Imidazole	502/502/502	50/50/50 $\mu$ L	10 sec	10 sec	10 sec
TCA	238/475/475	250/500/500 $\mu$ L	15 sec	15 sec	15 sec
Iodine	6.8/6.8/6.8	80/80/80 $\mu$ L	30 sec	30 sec	30 sec
Beaucage	34/51/51	80/120/120	100 sec	200 sec	200 sec
Acetonitrile	NA	1150/1150/1150 $\mu$ L	NA	NA	NA

- Wait time does not include contact time during delivery.

Table III: Nucleic acid sequences

RPI#	Name	Sequence	Seq. ID No.
15404	R-2.1 & 2.7	AAGCACUAAUGGAGA	1
17161	R-3.1	AAGCACUACAGUAA	2
15400	SM-2.1	UCUCCAU CUGAUGAGGCCGUUAGGCCGAA AGUGCUUG	3
17159	SM-2.7	UCUCCAU CUGAUGAGGCCGUUAGGCCGAA AGUGCUUG CGAGUG	4
17160	SM-3.1	UUACUGU CUGAUGAGGCCGUUAGGCCGAA AGUGCUUG CGAGUG	5
17162	s-2.1	caagcacuuucucaucagauaggaga	6
17163	s-2.2	cacucgcaagcacuuucucaucagauaggaga	7
17164	s-2.3	cacucgcaagcacccuaucaaggcagua	8
17165	s-2.4	cacucgcaagcacccuaucaagguggaga	9
15405	T-2a	UACUGCCUGAUAGGGUGCUUGCGAGUG	10

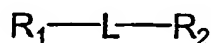
UPPER CASE = RIBO

lower case = 2'-O-methyl

Claims

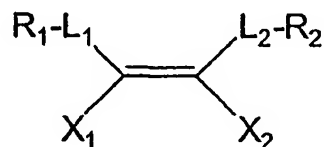
1. A nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components, wherein, in response to an interaction of a target signaling agent with the nucleic acid sensor molecule, the enzymatic nucleic acid component catalyzes a chemical reaction involving covalent attachment of at least a portion of a reporter molecule to the nucleic acid sensor molecule.
2. A method comprising the steps of:
  - a. contacting the nucleic acid sensor molecule and the reporter molecule of claim 1 with a system under conditions suitable for the enzymatic nucleic acid component of the nucleic acid sensor molecule to attach at least a portion of the reporter molecule to the nucleic acid sensor molecule in the presence of a target signaling agent; and
  - b. assaying for the attachment of the reporter molecule to the nucleic acid sensor molecule.
3. A nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components, wherein, in response to an interaction of a target signaling agent with the nucleic acid sensor molecule, the enzymatic nucleic acid component can carry out a chemical reaction involving isomerization of at least a portion of a reporter molecule.
4. A method, comprising the steps of:
  - a. contacting the nucleic acid sensor molecule and the reporter molecule of claim 3 with a system under conditions suitable for the enzymatic nucleic acid component of the nucleic acid sensor molecule to isomerize at least a portion of the reporter molecule in the presence of a target signaling agent; and
  - b. assaying for the isomerization reaction.
5. A nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components, wherein, in response to an interaction of a target signaling agent with the nucleic acid sensor molecule, the enzymatic component catalyses a a phosphorylation or dephosphorylation reaction on a non-oligonucleotide-based portion of a reporter molecule.
6. A method comprising the steps of:
  - a. contacting the nucleic acid sensor molecule and the reporter molecule of claim 5 with a system under conditions suitable for the enzymatic nucleic acid component

- of the nucleic acid sensor molecule to phosphorylate a non-oligonucleotide-based portion of the reporter molecule in the presence of a target signaling agent; and
- b. assaying for the phosphorylation reaction.
7. A method comprising the steps of:
    - a. contacting the nucleic acid sensor molecule and the reporter molecule of claim 5 with a system under conditions suitable for the enzymatic nucleic acid component of the nucleic acid sensor molecule to dephosphorylate a non-oligonucleotide-based portion of the reporter molecule in the presence of a target signaling agent; and
    - b. assaying for the dephosphorylation reaction.
  8. The nucleic acid sensor molecule of any of claims 1, 3, or 5, wherein said enzymatic nucleic acid component and the sensor component are distinct moieties.
  9. The nucleic acid sensor molecule of claim 8, wherein said enzymatic nucleic acid component and the sensor component are joined by a linker region.
  10. The nucleic acid sensor molecule of claim 1, wherein said reporter molecule comprises the formula:



wherein R1 is selected from the group consisting of alkyl, alkoxy, hydrogen, hydroxy, sulfhydryl, ester, anhydride, acid halide, amide, nitrile, phosphate, phosphonate, nucleoside, nucleotide, oligonucleotide; R2 is selected from the group consisting of molecular beacons, small molecules, fluorophores, chemophores, ionophores, radio-isotopes, photophores, peptides, proteins, enzymes, antibodies, nucleic acids, and enzymatic nucleic acids; L represents a linker which can be present or absent, and “-” represents a chemical bond

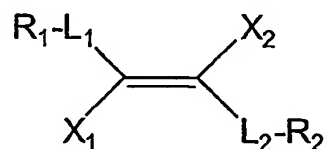
11. The nucleic acid sensor molecule of claim 3, wherein said reporter molecule comprises the formula:



wherein R1 and R2 each represent compounds, which can be the same or different, that generate a detectable signal or quench a detectable signal when an isomerization reaction is catalyzed, selected from the group consisting of molecular beacons, small molecules, fluorophores, chemophores, ionophores, radio-isotopes, photophores, peptides, proteins, enzymes, antibodies, nucleic acids, and enzymatic nucleic acids; L1 and L2 each

represent a linker which can be the same or different and which can be present or absent; X1 and X2 each represent an atom, compound, or molecule that can be the same or different, and “-“ represents a chemical bond.

12. The nucleic acid sensor molecule of claim 3, wherein said reporter molecule comprises the formula:



wherein R1 and R2 each represent compounds, which can be the same or different, that generate a detectable signal or quench a detectable signal when an isomerization reaction is catalyzed, selected from the group consisting of molecular beacons, small molecules, fluorophores, chemophores, ionophores, radio-isotopes, photophores, peptides, proteins, enzymes, antibodies, nucleic acids, and enzymatic nucleic acids; L1 and L2 each represent a linker which can be the same or different and which can be present or absent; X1 and X2 represent an atom, compound, or molecule that can be the same or different, and “-“ represents a chemical bond.

13. The method of any of claims 2, 4, 6, or 7, wherein the detection of a chemical reaction is indicative of the presence of the target signaling agent in the system.
14. The method of any of claims 2, 4, 6, or 7, wherein the absence of a chemical reaction is indicative of the system lacking the target signaling agent.
15. A method, comprising the steps of:
- a. contacting a nucleic acid sensor molecule which comprises: (i) an enzymatic nucleic acid component comprising a substrate binding region and a catalytic region; and (ii) a sensor component comprising a nucleic acid sequence that, upon interacting with a complementary sequence in the enzymatic nucleic acid component, inhibits the activity of the enzymatic nucleic acid component, and a reporter molecule comprising a nucleic acid sequence complementary to the substrate binding region of the enzymatic nucleic acid component of the nucleic acid sensor molecule with a system under conditions suitable for the enzymatic nucleic acid component of the nucleic acid sensor molecule to catalyze cleavage of the reporter molecule in the presence of a target signaling molecule; and
  - b. assaying for the cleavage reaction of step (a).
16. A method, comprising the steps of:

- a. contacting a nucleic acid sensor molecule which comprises: (i) an enzymatic nucleic acid component comprising a substrate binding region and a catalytic region; and (ii) a sensor component comprising a nucleic acid sequence that, upon interacting with a complementary sequence in the enzymatic nucleic acid component, inhibits the activity of the enzymatic nucleic acid component, and a reporter molecule comprising a nucleic acid sequence complementary to the substrate binding region of the enzymatic nucleic acid component of the nucleic acid sensor molecule with a system under conditions suitable for the enzymatic nucleic acid component of the nucleic acid sensor molecule to catalyze a ligation reaction involving the reporter molecule in the presence of a target signaling molecule; and
  - b. assaying for the ligation reaction in step (a).
17. The nucleic acid sensor molecule of any of claims 1, 3, or 5, wherein said nucleic acid sensor molecule is attached to a solid surface.
  18. The nucleic acid sensor molecule of claim 17, wherein the solid surface is selected from the group consisting of silicon-based chips, silicon-based beads, controlled pore glass, polystyrene, cross-linked polystyrene, nitrocellulose, biotin, plastics, metals and polyethylene films.
  19. The method of claim 16, wherein the ligation reaction causes at least a portion of the reporter molecule to be attached to the nucleic acid sensor molecule.
  20. The method of claim 15, wherein the cleavage of the reporter molecule is indicative of the presence of the target signaling molecule in the system.
  21. The method of claim 15, wherein the absence of cleavage of the reporter molecule is indicative of the system lacking the target signaling molecule.
  22. The method of claim 16, wherein the ligation reaction causes at least a portion of the reporter molecule to be attached to a separate molecule.
  23. The method of claim 16, wherein the ligation of the reporter molecule is indicative of the presence of the target signaling molecule in the system.
  24. The method of claim 16, wherein the absence of ligation of the reporter molecule is indicative of the system lacking the target signaling molecule.
  25. The method of any of claims 15 or 16, wherein the system is an *in vitro* system.
  26. The method of claim 25, wherein the *in vitro* system is a sample derived from the group consisting of a patient, plant, water, beverage, food preparation, and soil.

27. The method of claims 11 or 12, wherein the target signaling molecule is an RNA, DNA, analog of RNA or analog of DNA.
28. The method of claims 11 or 12, wherein the target signaling molecule is an RNA derived from a bacteria, virus, fungi, plant or mammalian genome.
29. The method of any of claims 2, 4, 6, 7, 15, or 16, wherein the enzymatic nucleic acid component of said nucleic acid sensor molecule is selected from the group consisting of hammerhead, hairpin, inozyme, G-cleaver, Zinzyme, RNase P EGS nucleic acid and Amberzyme motif.
30. The method of any of claims 2, 4, 6, 7, 15, or 16, wherein the enzymatic nucleic acid component of said nucleic acid sensor molecule is a DNAzyme.
31. The method of any of claims 2, 4, 6, 7, 15, or 16, wherein the reporter molecule comprises a detectable label selected from the group consisting of chromogenic substrate, fluorescent labels, chemiluminescent labels, and radioactive labels.
32. The method of any of claims 2, 4, 6, 7, 15, or 16, wherein the reporter molecule is immobilized on a solid support.
33. The method of any of claims 2, 4, 6, 7, 15, or 16, wherein the sensor component of the nucleic acid sensor molecule is RNA, DNA, analog of RNA or analog of DNA.
34. The method of any of claims 2, 4, 6, 7, 15, or 16, wherein the sensor component of the nucleic acid sensor molecule is covalently linked to the nucleic acid sensor molecule by a linker.
35. The method of claim 35, wherein the linker is selected from the group consisting of one or more nucleotides, abasic moiety, polyether, polyamine, polyamide, peptide, carbohydrate, lipid, and polyhydrocarbon compounds, and any combination thereof.
36. The method of claim 15 or claim 16, wherein the sensor component of the nucleic acid sensor molecule is not covalently linked to the nucleic acid sensor molecule.
37. The method of claim 15 or claim 16, wherein the reporter molecule is RNA, DNA, RNA analog, or DNA analog.
38. A kit comprising:
  - (a) a nucleic acid sensor molecule which comprises: (i) an enzymatic nucleic acid component comprising a substrate binding region and a catalytic region; and (ii) a sensor component comprising a nucleic acid which inhibits the activity of the enzymatic nucleic acid component upon interacting with a complementary sequence in the enzymatic nucleic acid component; and

- (b) a reporter molecule cleavable by the enzymatic nucleic acid component of the nucleic acid sensor molecule in the presence of a target signaling molecule, wherein the reporter molecule comprises a chemical moiety capable of emitting a detectable signal upon cleavage of the reporter molecule.
39. A kit comprising:
- (a) a nucleic acid sensor molecule comprising an enzymatic nucleic acid component including one or more sensor components; and
  - (b) a reporter molecule, wherein, in response to an interaction of a target signaling molecule with the nucleic acid sensor molecule, the enzymatic nucleic acid component of the nucleic acid sensor molecule catalyzes a chemical reaction involving covalent attachment of at least a portion of a reporter molecule to the nucleic acid sensor molecule.
40. A kit comprising:
- (a) a nucleic acid sensor molecule comprising an enzymatic nucleic acid component including one or more sensor components; and
  - (b) a reporter molecule, wherein, in response to an interaction of a target signaling molecule with the nucleic acid sensor molecule, the enzymatic nucleic acid component can carry out a chemical reaction involving isomerization of at least a portion of a reporter molecule.
41. A kit comprising:
- (a) a nucleic acid sensor molecule comprising an enzymatic nucleic acid component including one or more sensor components; and
  - (b) a reporter molecule including a non-oligonucleotide-based portion, wherein, in response to an interaction of a target signaling molecule with the nucleic acid sensor molecule, the enzymatic nucleic acid component catalyses a chemical reaction involving phosphorylation of a non-oligonucleotide-based portion of a reporter molecule.
42. A kit comprising:
- (a) a nucleic acid sensor molecule comprising an enzymatic nucleic acid component including one or more sensor components; and
  - (b) a reporter molecule including a non-oligonucleotide-based portion, wherein, in response to an interaction of a target signaling molecule with the nucleic acid sensor molecule, the enzymatic component catalyses a chemical reaction



involving dephosphorylation of a non-oligonucleotide-based portion of a reporter molecule.

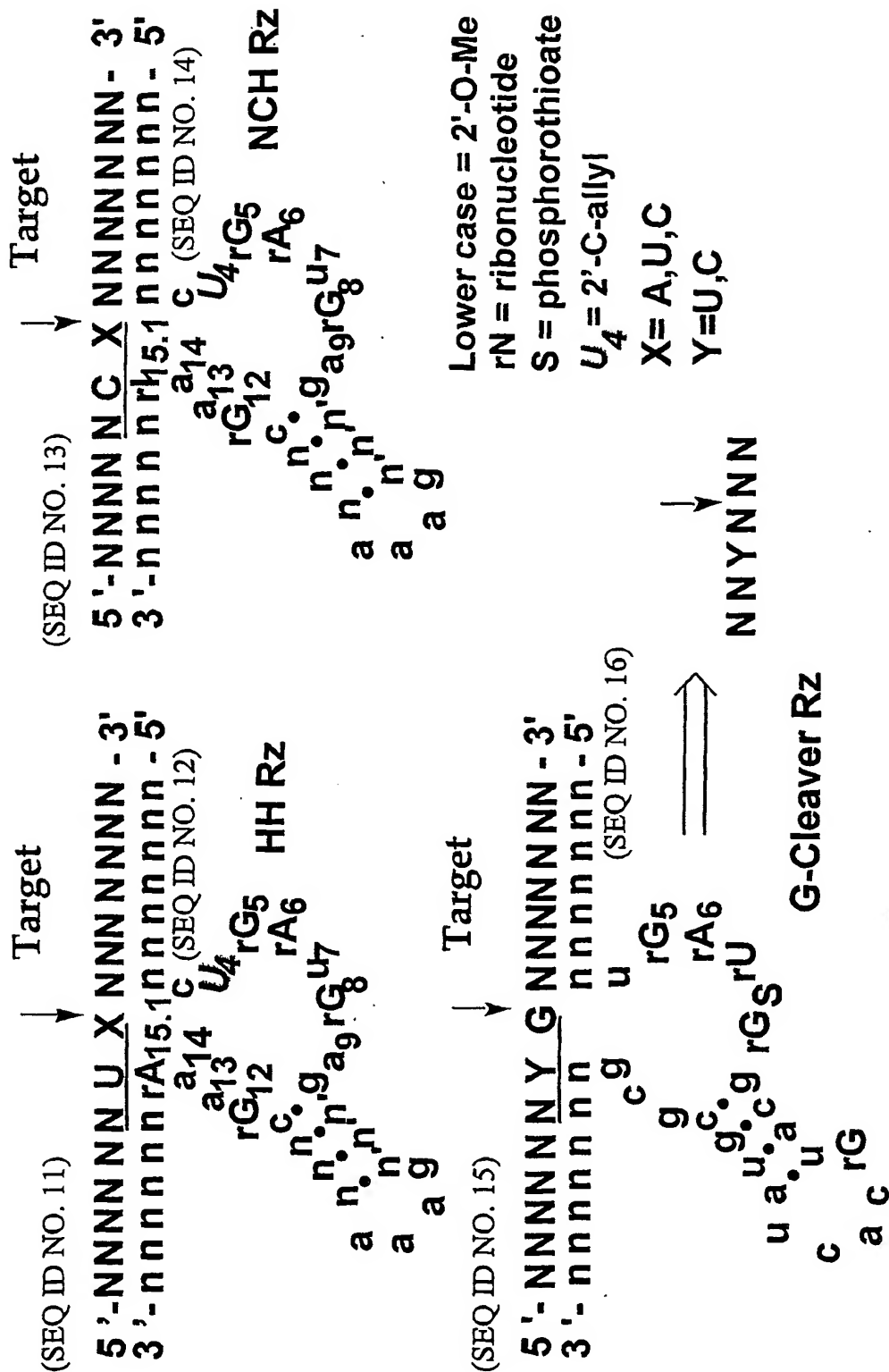
43. A method comprising the step of contacting one or more components of the kit of claim 38 with a system under conditions suitable for at least a portion of the reporter molecule in the kit to be cleaved by the nucleic acid sensor molecule in the kit in the presence of a target signaling molecule.
44. A method comprising the step of contacting one or more components of the kit of claim 39 with a system under conditions suitable for at least a portion of the reporter molecule in the kit to be covalently attached to the nucleic acid sensor molecule in the kit in the presence of a target signaling molecule.
45. A method comprising the step of contacting one or more components of the kit of claim 40 with a system under conditions suitable for at least a portion of the reporter molecule in the kit to be isomerized by the nucleic acid sensor molecule in the kit in the presence of a target signaling molecule.
46. A method comprising the step of contacting one or more components of the kit of claim 41 with a system under conditions suitable for at least a portion of the reporter molecule in the kit to be phosphorylated by the nucleic acid sensor molecule in the kit in the presence of a target signaling molecule.
47. A method comprising the step of contacting one or more components of the kit of claim 42 with a system under conditions suitable for at least a portion of the reporter molecule in the kit to be dephosphorylated by the nucleic acid sensor molecule in the kit in the presence of a target signaling molecule.
48. The kit of any of claims 38, 39, 40, 41, or 42, wherein the target signaling molecule is an RNA derived from a bacteria, virus, fungi, plant or mammalian genome.
49. The kit of any of claims 38, 39, 40, 41, or 42, wherein the enzymatic nucleic acid component of said nucleic acid sensor molecule is selected from the group consisting of hammerhead, hairpin, inozyme, G-cleaver, Zinzyme, RNase P, EGS nucleic acid and Amberzyme motif.
50. The kit of any of claims 38, 39, 40, 41, or 42, wherein the enzymatic nucleic acid component of said nucleic acid sensor molecule is a DNAzyme.
51. The kit of claim 38, wherein the chemical moiety capable of emitting a detectable signal is selected from the group consisting of chromogenic substrate, fluorescent labels, chemiluminescent labels, and radioactive labels.

52. The kit of any of claims 38, 39, 40, 41, or 42, wherein the reporter molecule is immobilized on a solid support.
53. The kit of any of claims 38, 39, 40, 41, or 42, wherein the sensor component of the nucleic acid sensor molecule is RNA, DNA, analog of RNA or analog of DNA.
54. The kit of any of claims 38, 39, 40, 41, or 42, wherein the sensor component of the nucleic acid sensor molecule is covalently linked to the nucleic acid sensor molecule by a linker.
55. The kit of any of claims 54, wherein the linker is selected from the group consisting of one or more nucleotides, abasic moiety, polyether, polyamine, polyamide, peptide, carbohydrate, lipid, and polyhydrocarbon compounds, and any combination thereof.
56. The kit of any of claims 38, 39, 40, 41, or 42, wherein the sensor component of the nucleic acid sensor molecule is not covalently linked to the nucleic acid sensor molecule.
57. The kit of any of claims 38, 39, 40, 41, or 42, wherein the reporter molecule is a RNA, DNA, RNA analog, or DNA analog.
58. A nucleic acid circuit comprising a nucleic acid sensor molecule which comprises an enzymatic nucleic acid component and one or more sensor components, wherein, in response to an interaction of a target signaling agent with the nucleic acid sensor molecule, the enzymatic nucleic acid component catalyzes a chemical reaction involving ligation of at least a portion of a nucleic acid based-component of the nucleic acid circuit.
59. A nucleic acid circuit comprising a nucleic acid sensor molecule which comprises an enzymatic nucleic acid component and one or more sensor components, wherein, in response to an interaction of a target signaling agent with the nucleic acid sensor molecule, the enzymatic nucleic acid component catalyzes a chemical reaction involving cleavage of at least a portion of a nucleic acid based-component of the nucleic acid circuit.
60. A nucleic acid computer comprising one or more nucleic acid circuits which comprises a nucleic acid sensor molecule comprising an enzymatic nucleic acid component and one or more sensor components, wherein, in response to an interaction of a target signaling agent with the nucleic acid sensor molecule, the enzymatic nucleic acid component catalyzes a chemical reaction involving ligation of at least a portion of a nucleic acid based-component of the nucleic acid circuit.
61. A nucleic acid computer comprising one or more nucleic acid circuits which comprises a nucleic acid sensor molecule including an enzymatic nucleic acid component and one or more sensor components, wherein, in response to an interaction of a target signaling

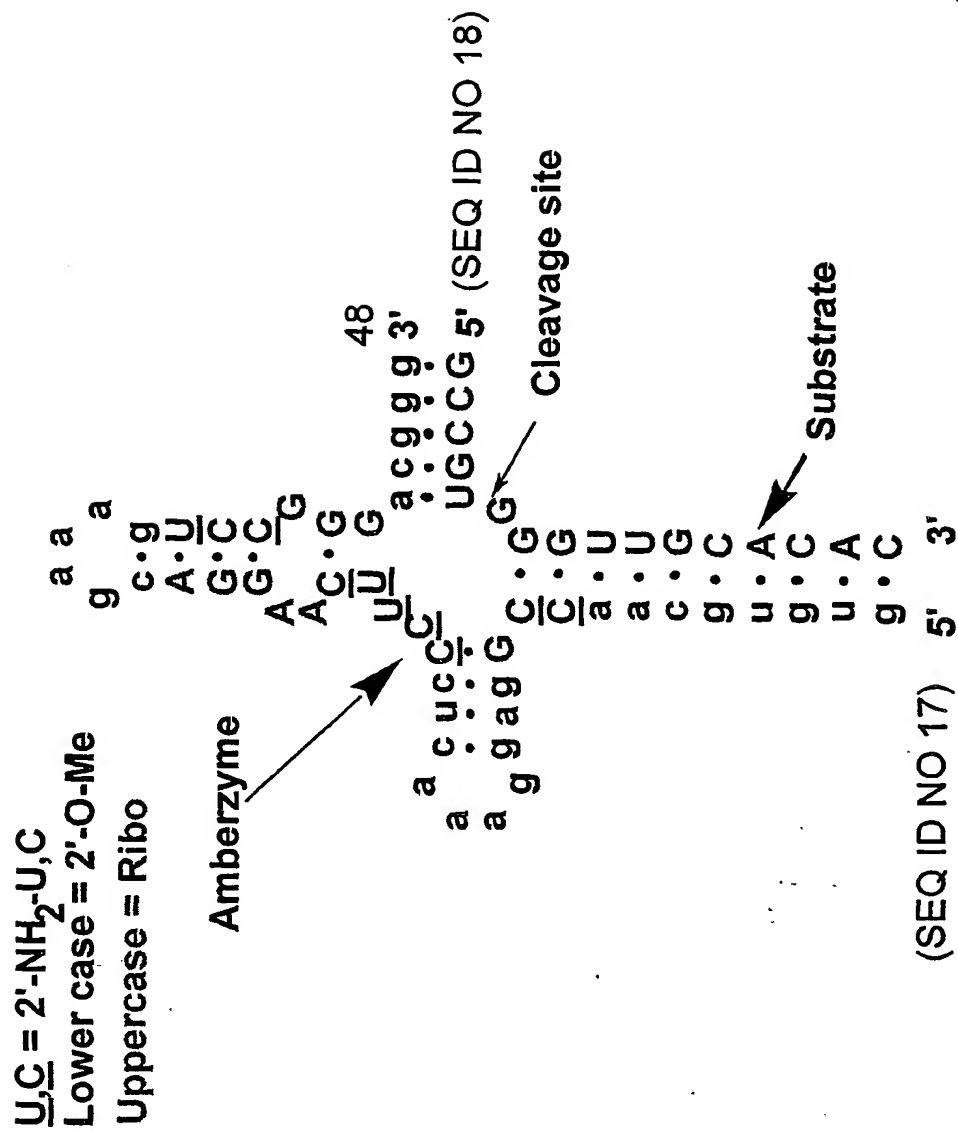
- agent with the nucleic acid sensor molecule, the enzymatic nucleic acid component catalyzes a chemical reaction involving cleavage of at least a portion of a nucleic acid based-component of the nucleic acid circuit.
62. The nucleic acid circuit of claim 58 or 59, wherein the target signaling agent is current.
  63. The nucleic acid circuit of claim 58 or 59, wherein the target signaling agent is voltage
  64. The nucleic acid circuit of claims 58 or 59, wherein the target signaling agent is impedance.
  65. The nucleic acid computer of claim 60 or 61, wherein the nucleic acid computer comprises a plurality of nucleic acid circuits that are arranged in a parallel array.
  66. The nucleic acid computer of claim 60 or 61, wherein the nucleic acid computer is used to detect a target signaling agent.
  67. The nucleic acid computer of claim 60 or 61, wherein the nucleic acid computer is used to provide a desired output.
  68. A method comprising the steps of:
    - (a) contacting the nucleic acid circuit of claim 58 with a target signaling agent under conditions suitable for the nucleic acid sensor molecule to ligate at least a portion of said nucleic acid circuit; and
    - (b) assaying the ligation in step (a).
  69. A method, comprising the steps of:
    - (a) contacting the nucleic acid circuit of claim 59 with a target signaling agent under conditions suitable for the nucleic acid sensor molecule to cleave at least a portion of a nucleic acid based-component of the nucleic acid circuit; and
    - (b) assaying the cleavage in step (a).
  70. The method of claim 68 or 69, wherein step (b) involves measuring current.
  71. The method of claim 68 or 69, wherein step (b) involves measuring voltage.
  72. The method of claim 68 or 69, wherein step (b) involves measuring capacitance.
  73. The method of claim 68 or 69, wherein step (b) involves measuring current.
  74. A method for isolating a nucleic acid sensor molecule of claim 1 comprising the steps of:
    - (a) contacting a random pool of nucleic acids with a target signaling molecule and a reporter molecule; and
    - (b) selecting for a nucleic acid sensor molecule that can catalyze a chemical reaction involving covalent attachment of at least a portion of said reporter molecule to the nucleic acid sensor molecule in the presence of the target signaling molecule.
  75. A method for isolating a nucleic acid sensor molecule of claim 3, comprising the steps of:

- (a) contacting a random pool of nucleic acids with a target signaling molecule and a reporter molecule; and
  - (b) selecting for a nucleic acid sensor molecule that can catalyze a chemical reaction involving ligation of at least a portion of said reporter molecule to the nucleic acid sensor molecule in the presence of the target signaling molecule.
76. A method for isolating a nucleic acid sensor molecule of claim 5, comprising the steps of:
- (a) contacting a random pool of nucleic acids with a target signaling molecule and a non-oligonucleotide-based reporter molecule; and
  - (b) selecting for a nucleic acid sensor molecule that can catalyze a chemical reaction involving phosphorylation of a non-oligonucleotide-based portion of said reporter molecule by the nucleic acid sensor molecule in the presence of the target signaling molecule.
77. A method for isolating a nucleic acid sensor molecule of claim 5, comprising the steps of:
- (a) contacting a random pool of nucleic acids with a target signaling molecule and a non-oligonucleotide-based reporter molecule; and
  - (b) selecting for a nucleic acid sensor molecule that can catalyze a chemical reaction involving dephosphorylation of a non-oligonucleotide-based portion of said reporter molecule by the nucleic acid sensor molecule in the presence of the target signaling molecule.
78. The method of claim 32, wherein said solid support is selected from the group consisting of silicon-based chips, silicon-based beads, controlled pore glass, polystyrene, cross-linked polystyrene, nitrocellulose, biotin, plastics, metals and polyethylene films.
79. The kit of claim 52, wherein said solid support is selected from the group consisting of silicon-based chips, silicon-based beads, controlled pore glass, polystyrene, cross-linked polystyrene, nitrocellulose, biotin, plastics, metals and polyethylene films.
80. The method of any of claims 2, 4, 6, 7, 15, 16, 17, 18, 43, 44, 45, 46, 47, 68, 69, 74, 75, 76, or 77, wherein said method is repeated at least one time..

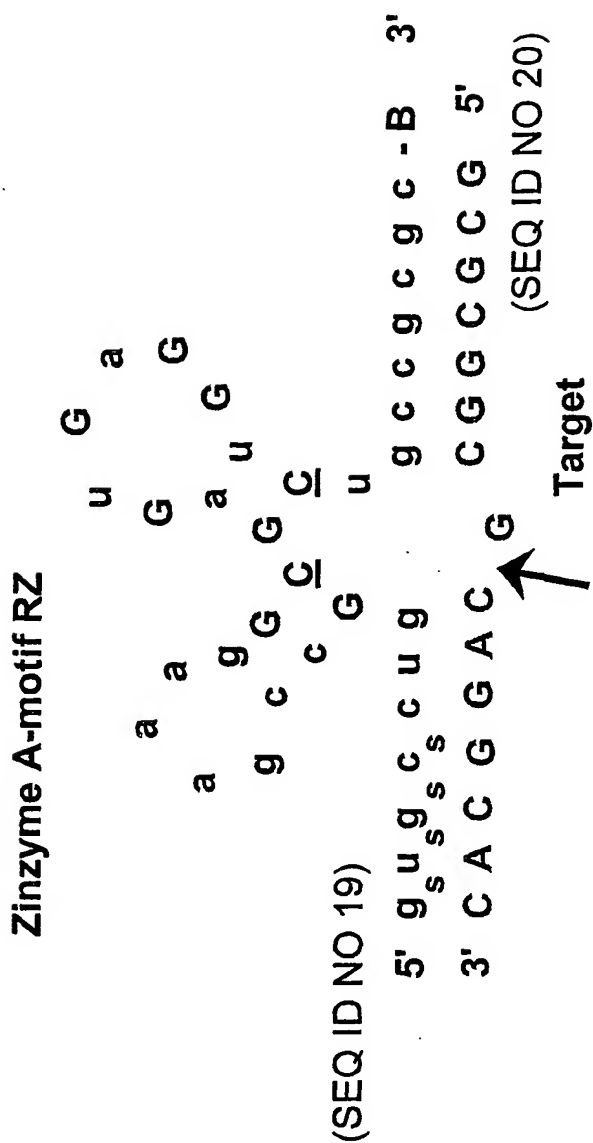
Figure 1: Examples of Nuclease Stable Ribozyme Motifs



**Figure 2: 2'-O-Me substituted Amberzyme Enzymatic Nucleic Acid Motif**



**Figure 3: Stabilized Zinzyme Ribozyme Motif**



**Legend**

Uppercase indicates natural ribo residues

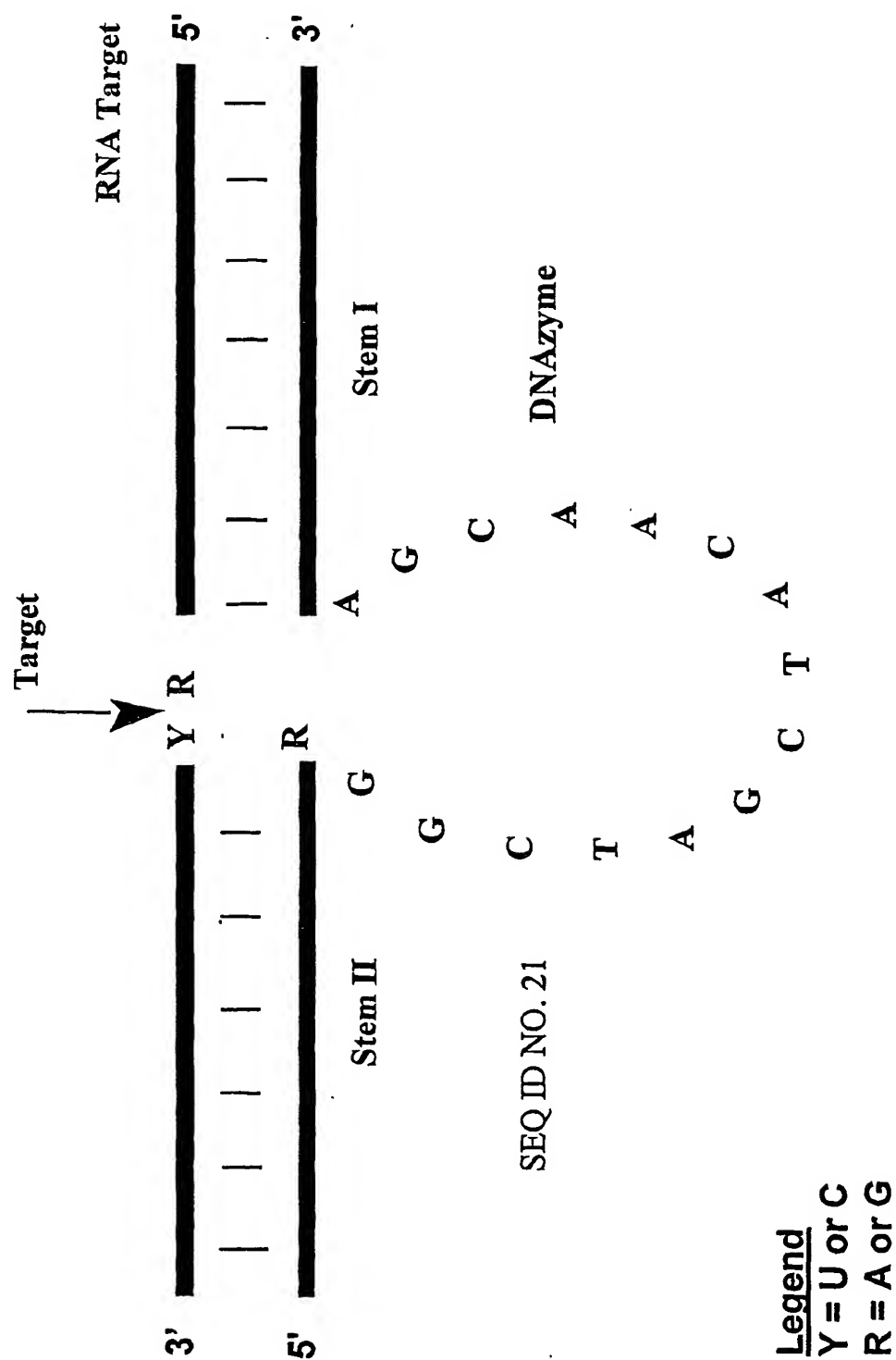
C indicates 2'-d-NH<sub>2</sub>-C

Lowercase: 2'-O-Me

Subscript<sub>s</sub> indicates phosphothioate linkage

B: 3'-3' abasic moiety

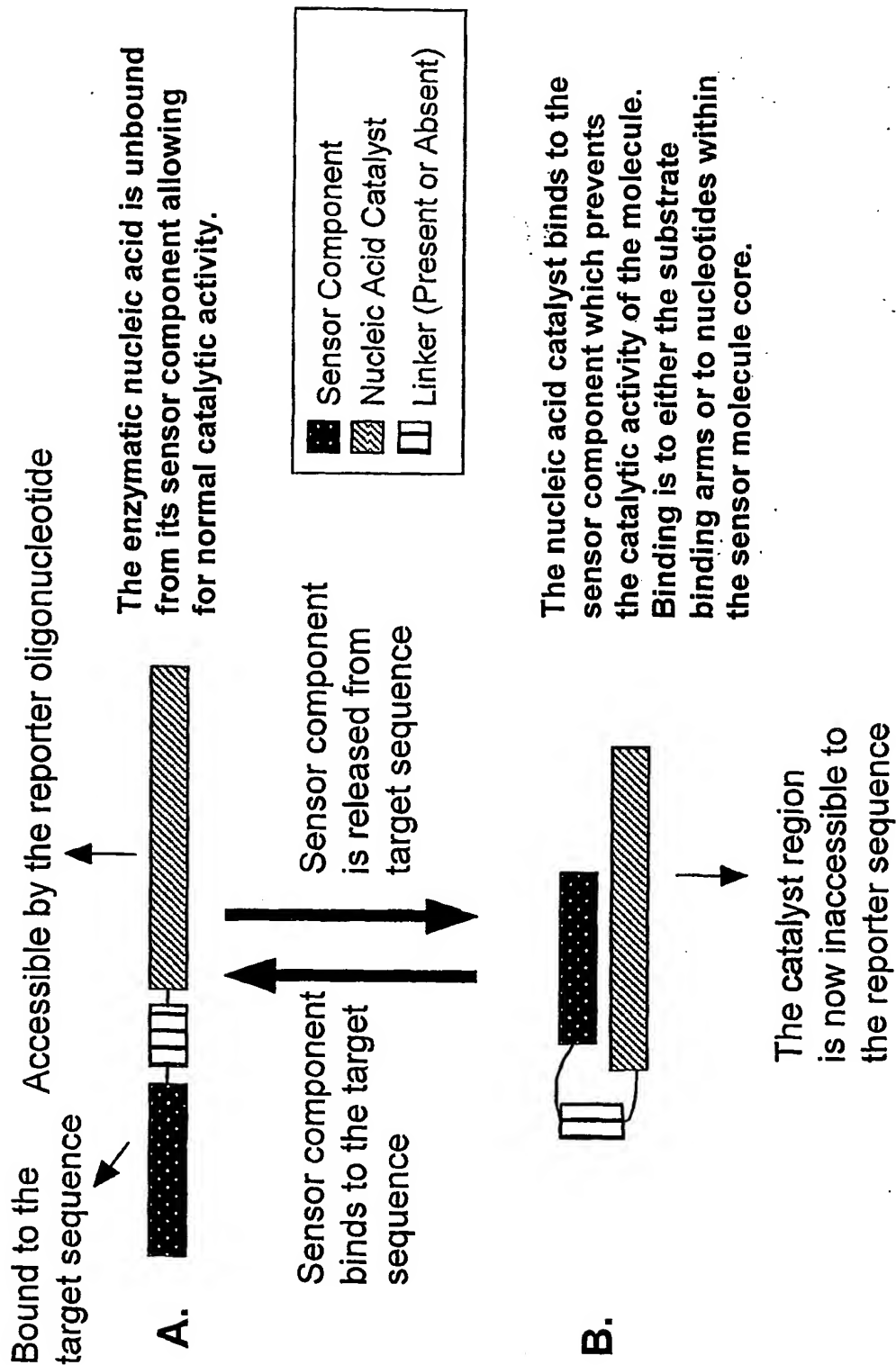
**Figure 4: DNAAzyme Motif**



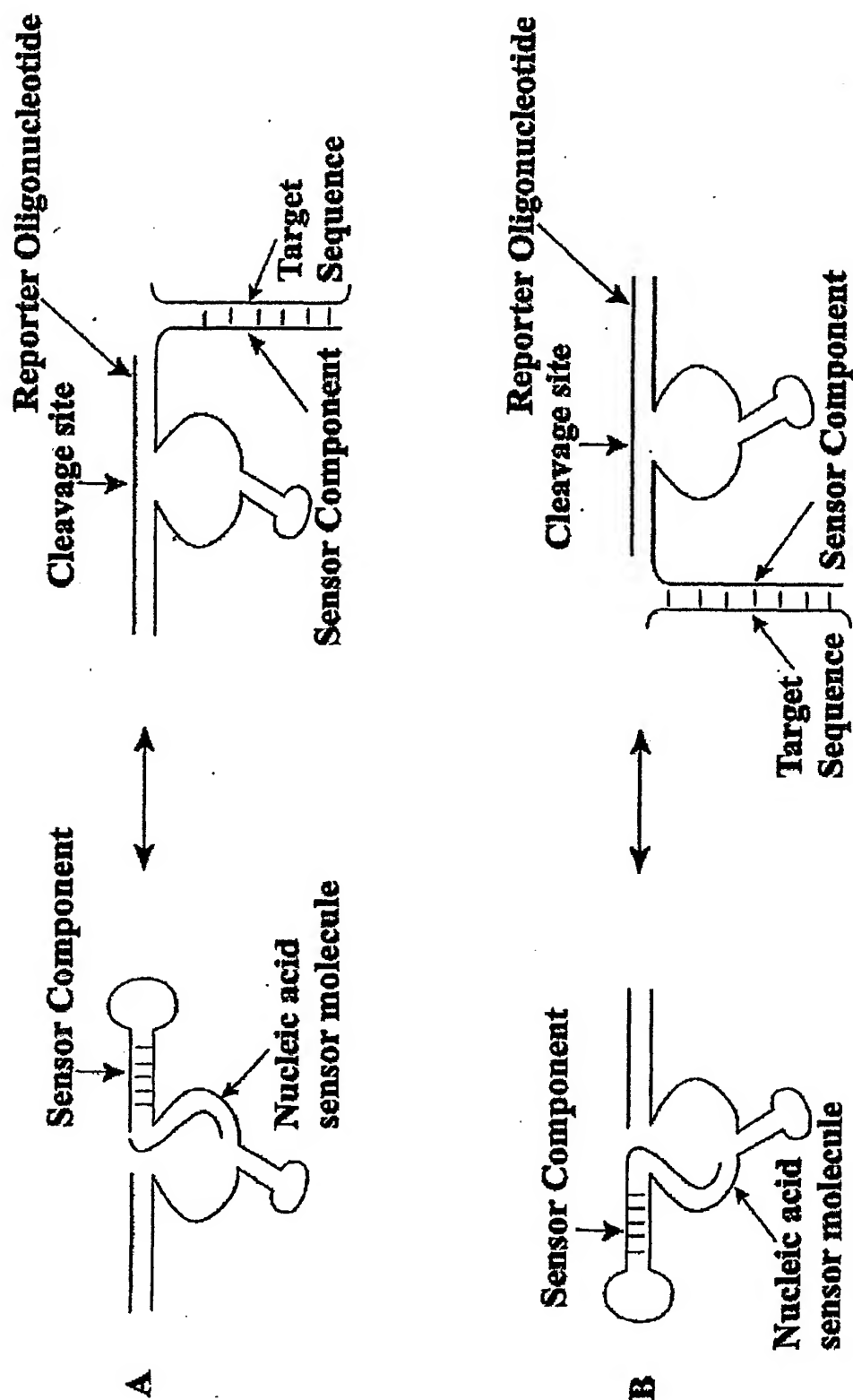




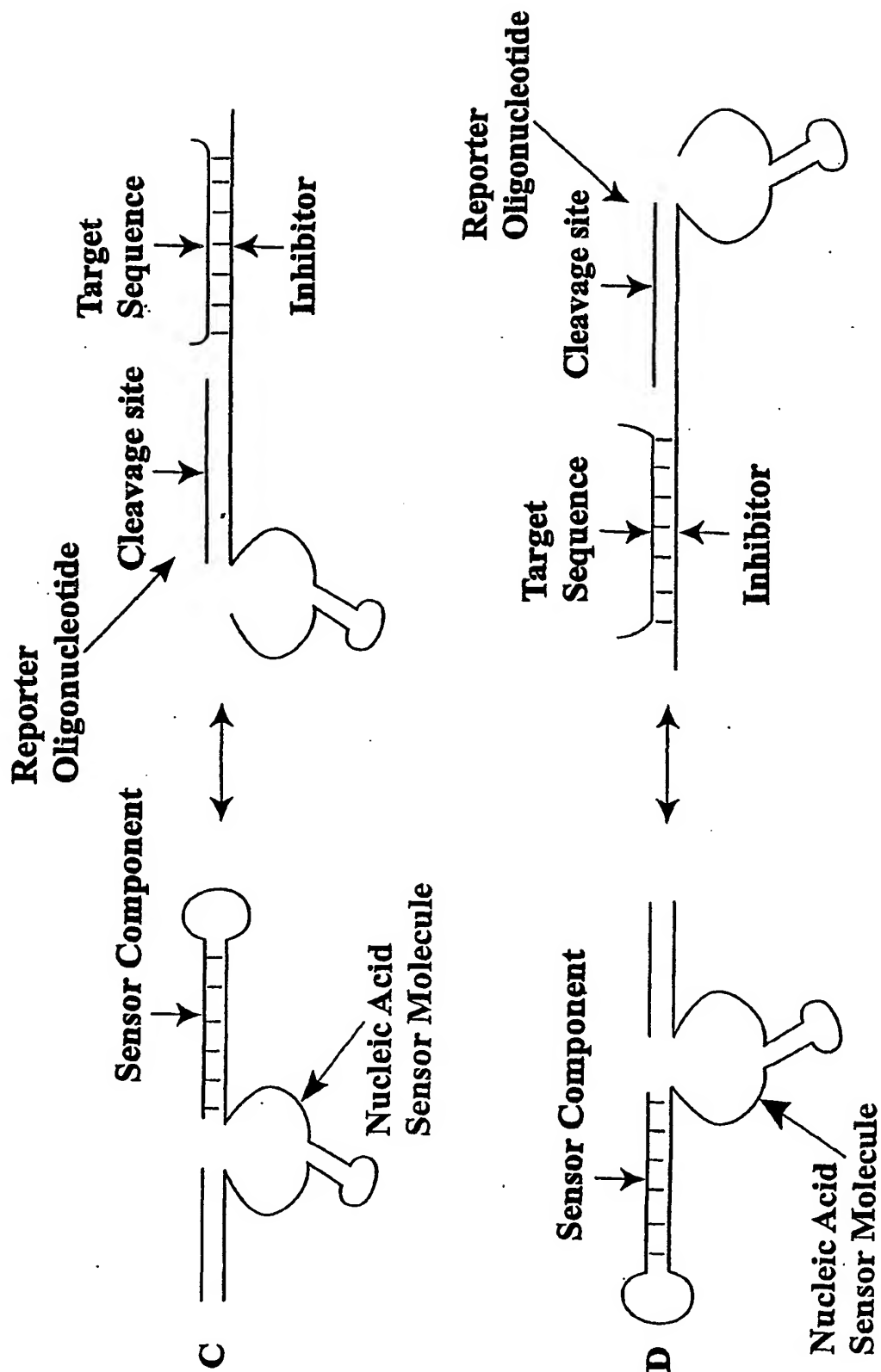
**Figure 6. Schematic Diagram Representing the Two Primary Configurations of the Diagnostic effector molecule**



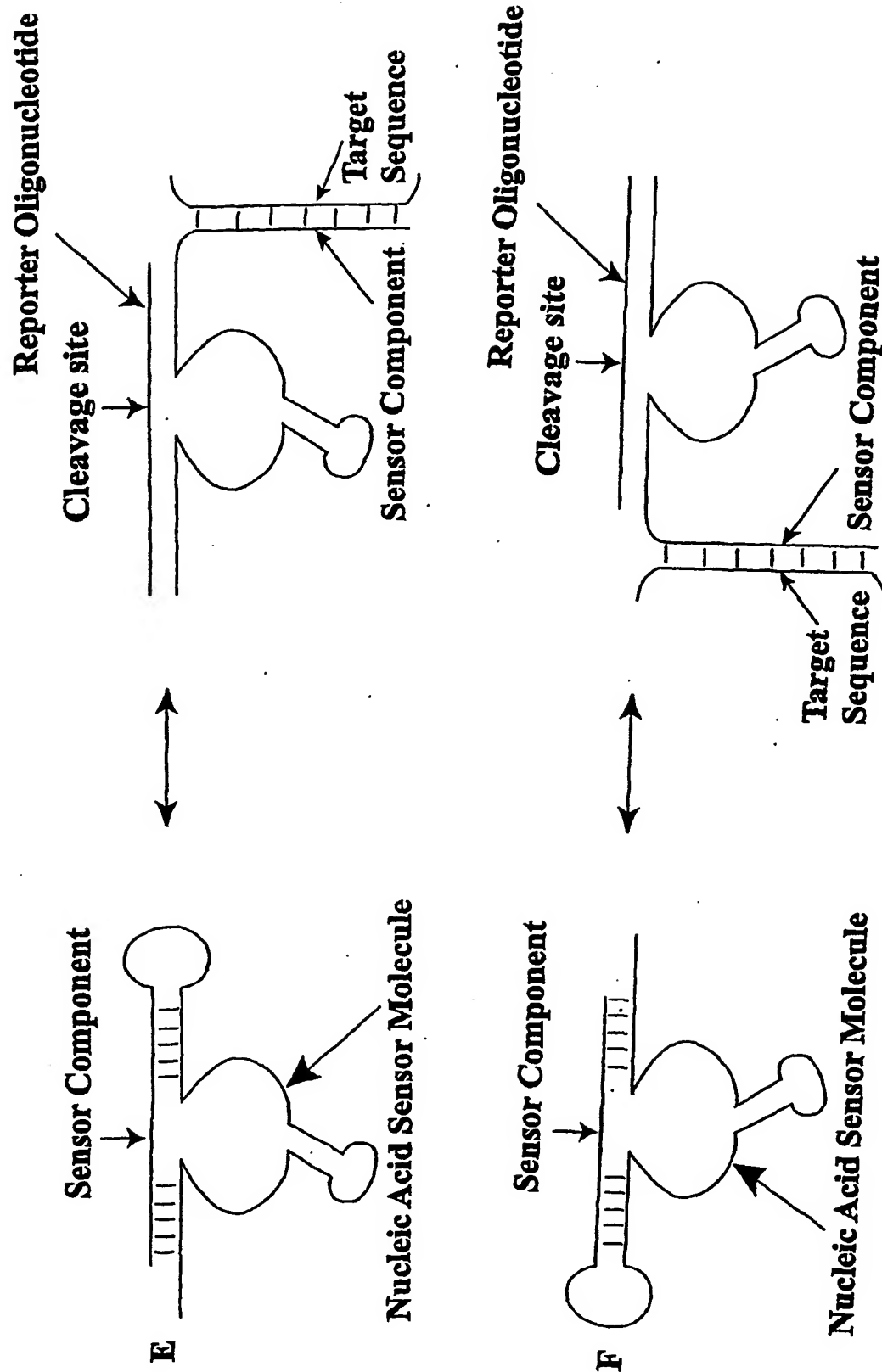
**Figure 7a. Examples of Diagnostic Effector Molecules**



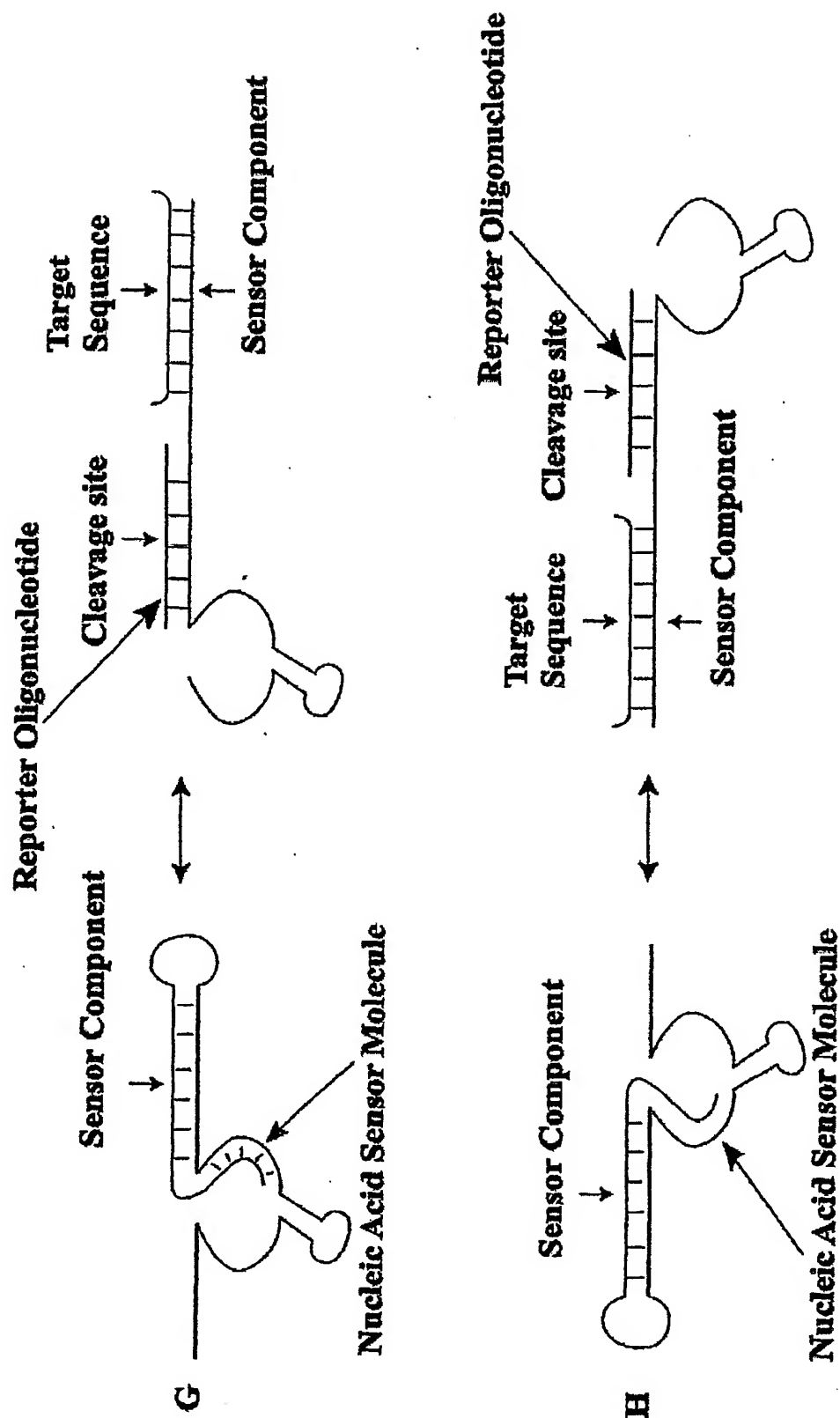
**Figure 7b. Examples of Diagnostic Effector Molecules**

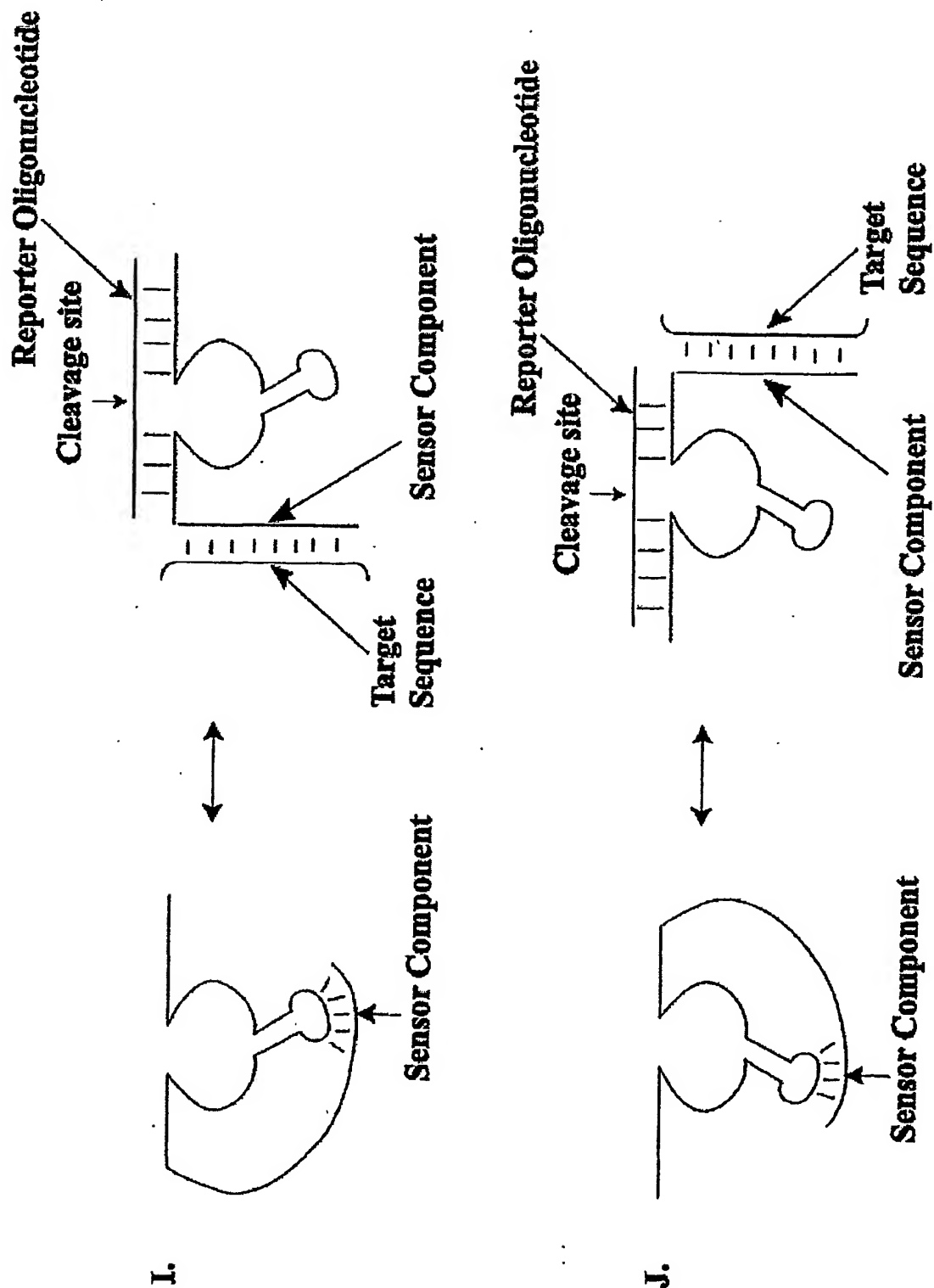


*Figure 8a. Examples of Diagnostic Effector Molecules*

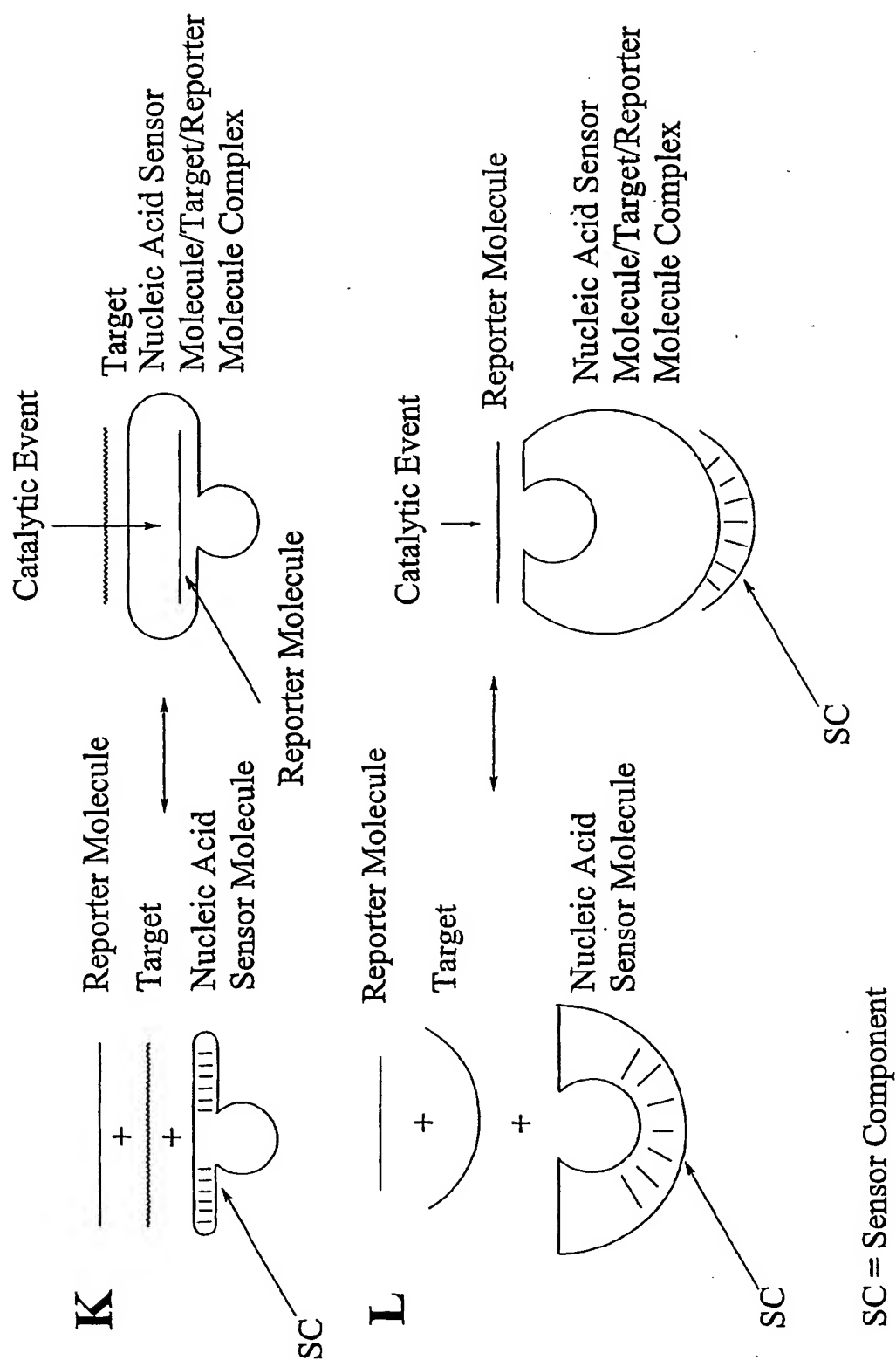


**Figure 8b. Examples of Diagnostic Effector Molecules**



**Figure 9. Examples of Diagnostic Effector Molecules**

**Figure 10: Examples of Diagnostic Effector Molecules**





**Figure 11: Examples of Diagnostic Effector Molecules**

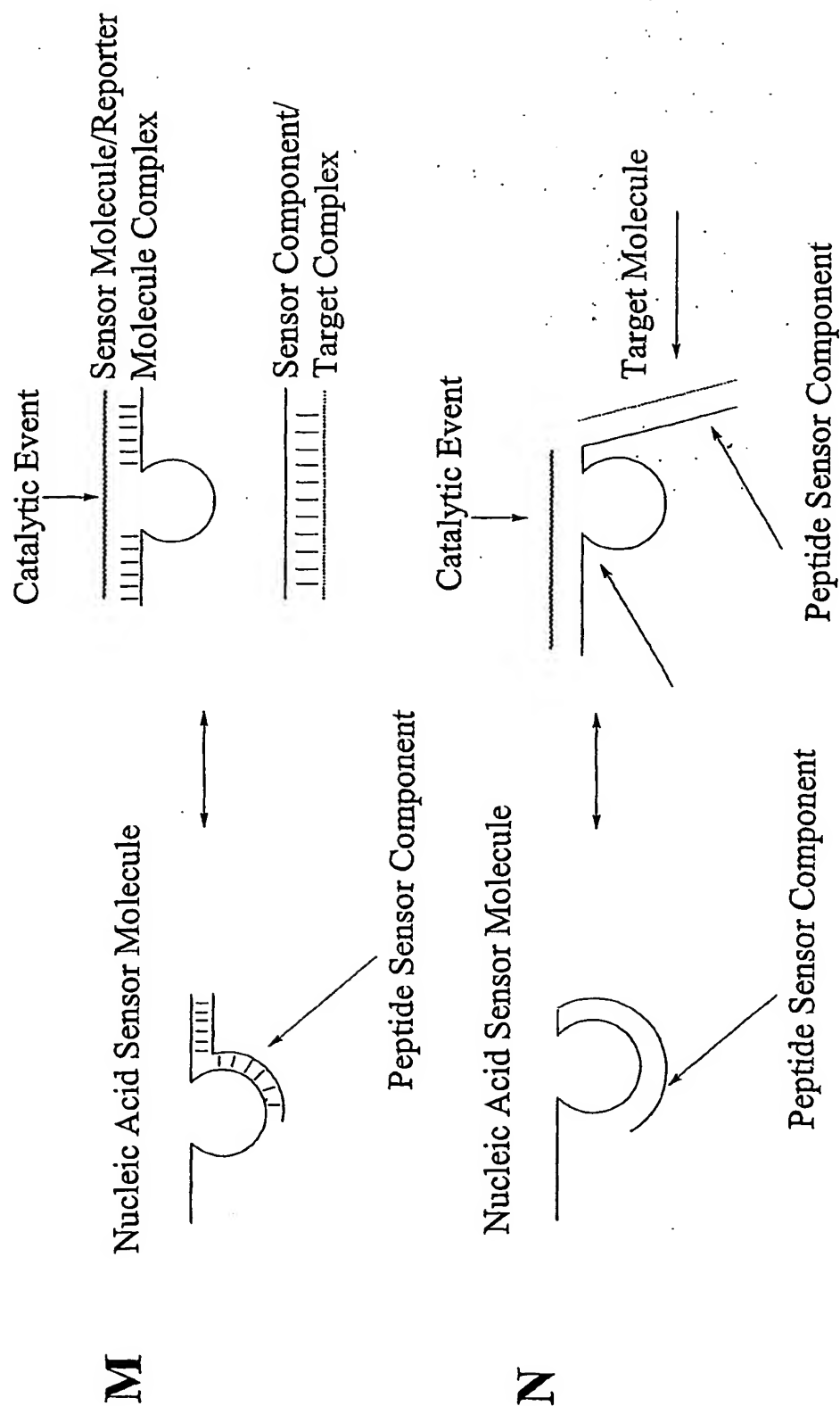
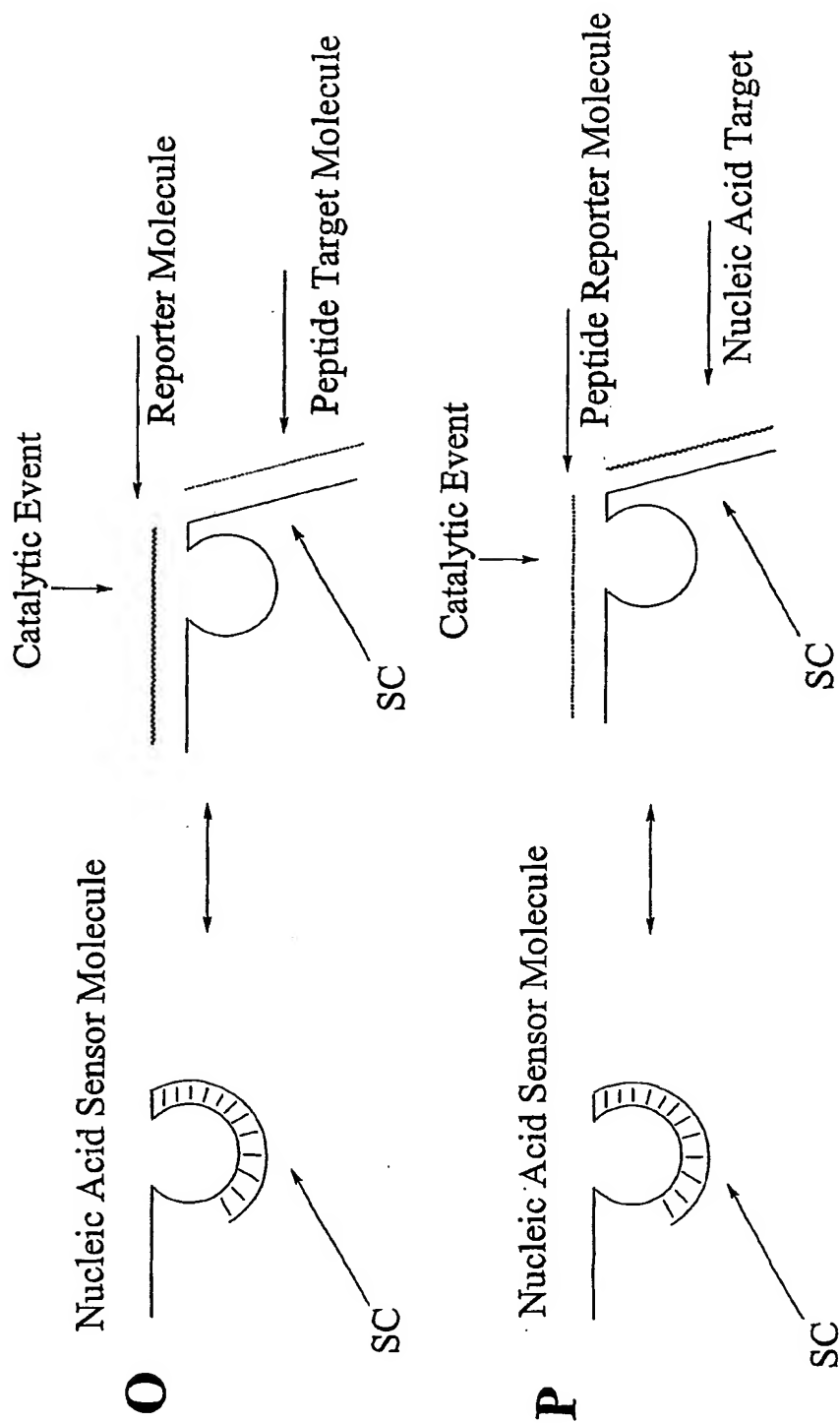
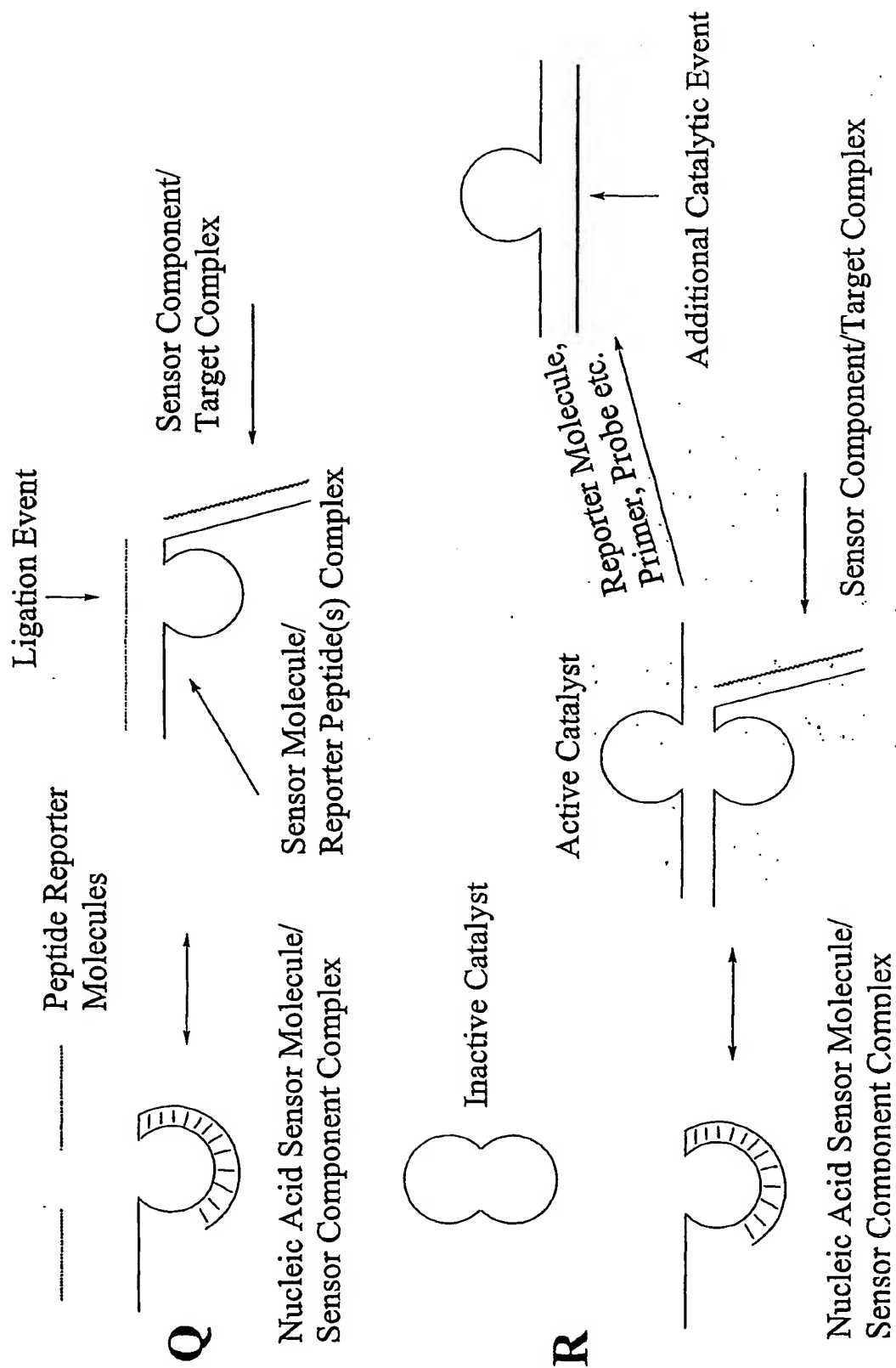


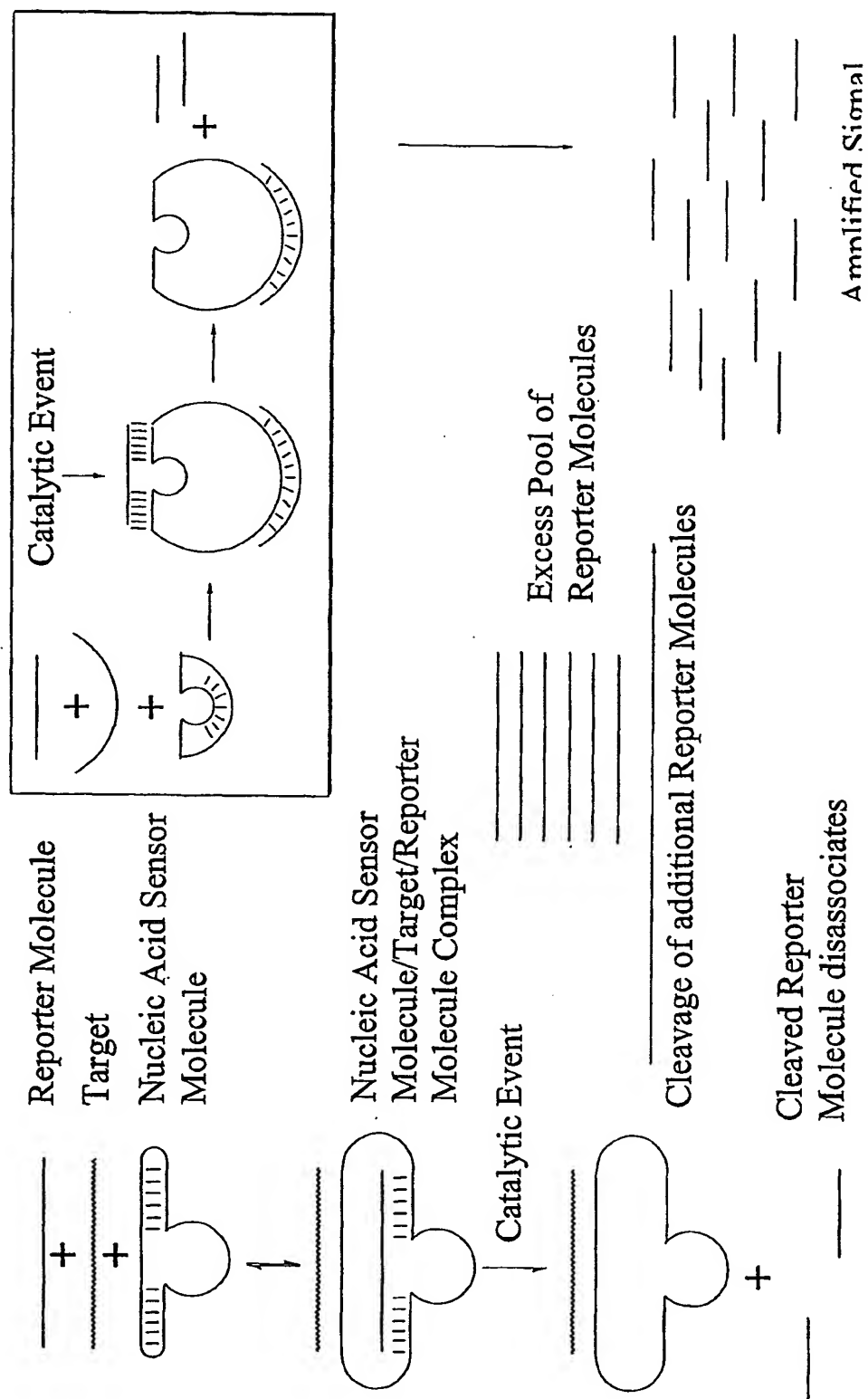
Figure 12: Examples of Diagnostic Effector Molecules



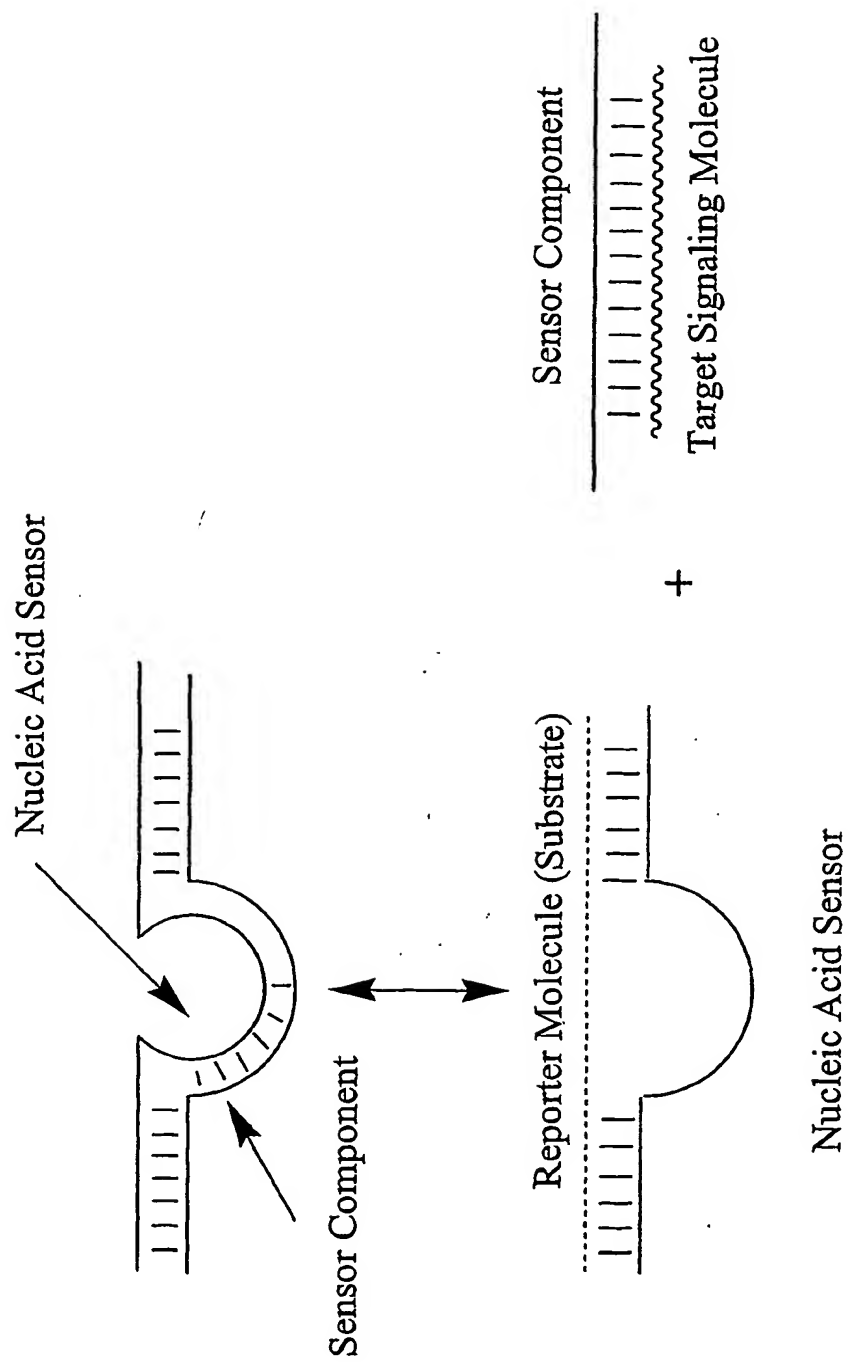
**Figure 13: Examples of Diagnostic Effector Molecules**



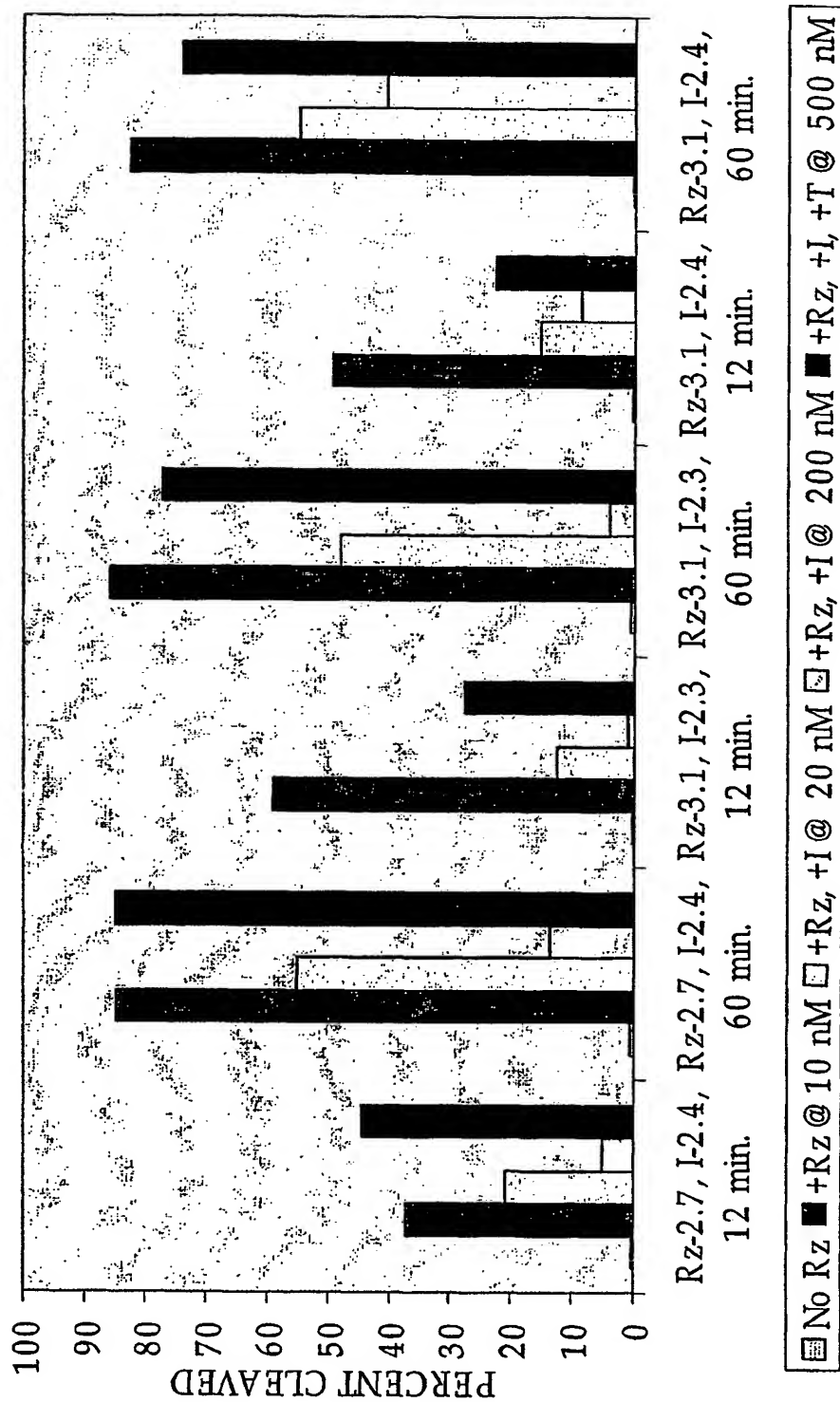
**Figure 14: Inherent Amplification of Signal**



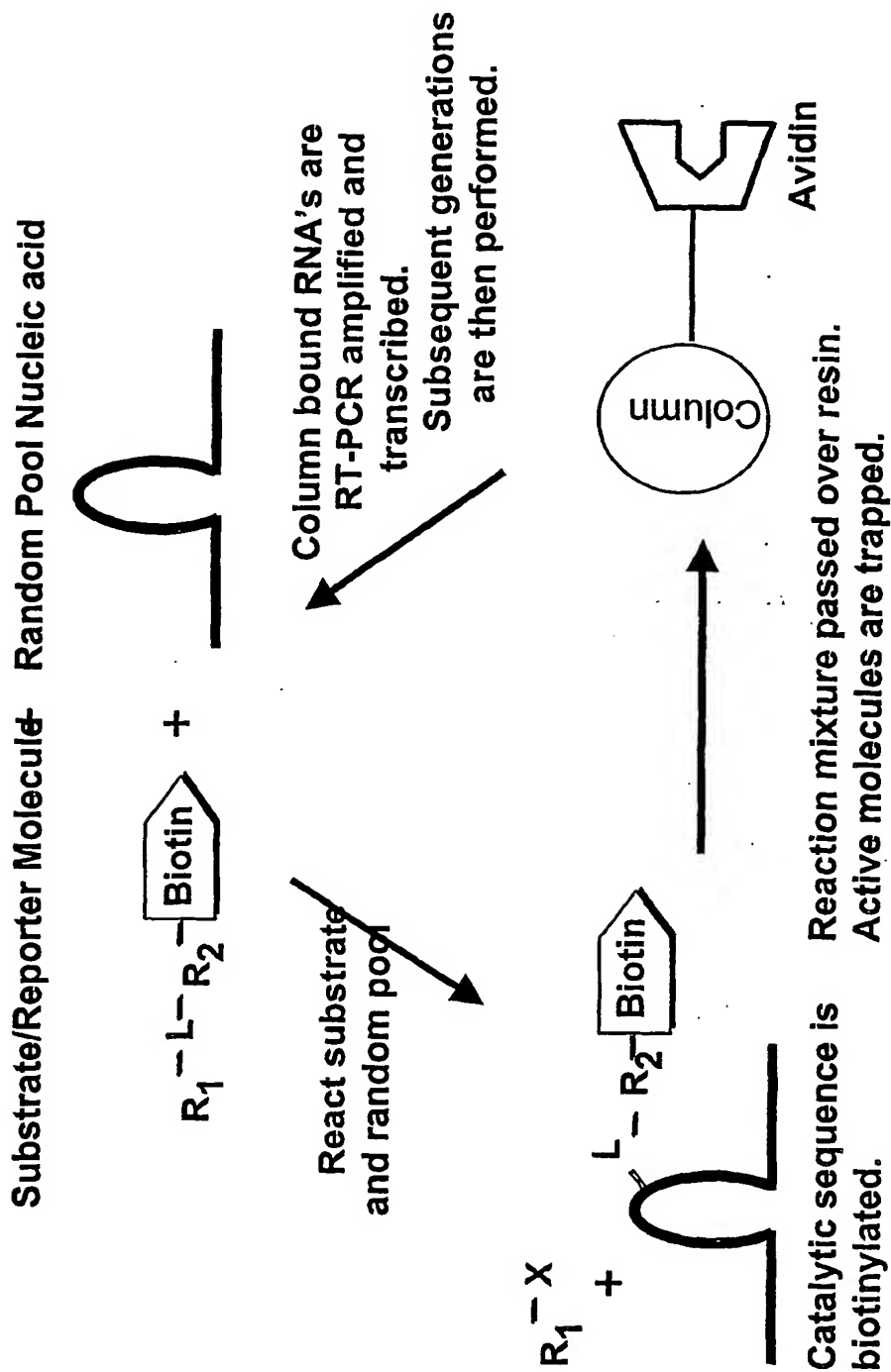
**Figure 15: Example of Diagnostic System**



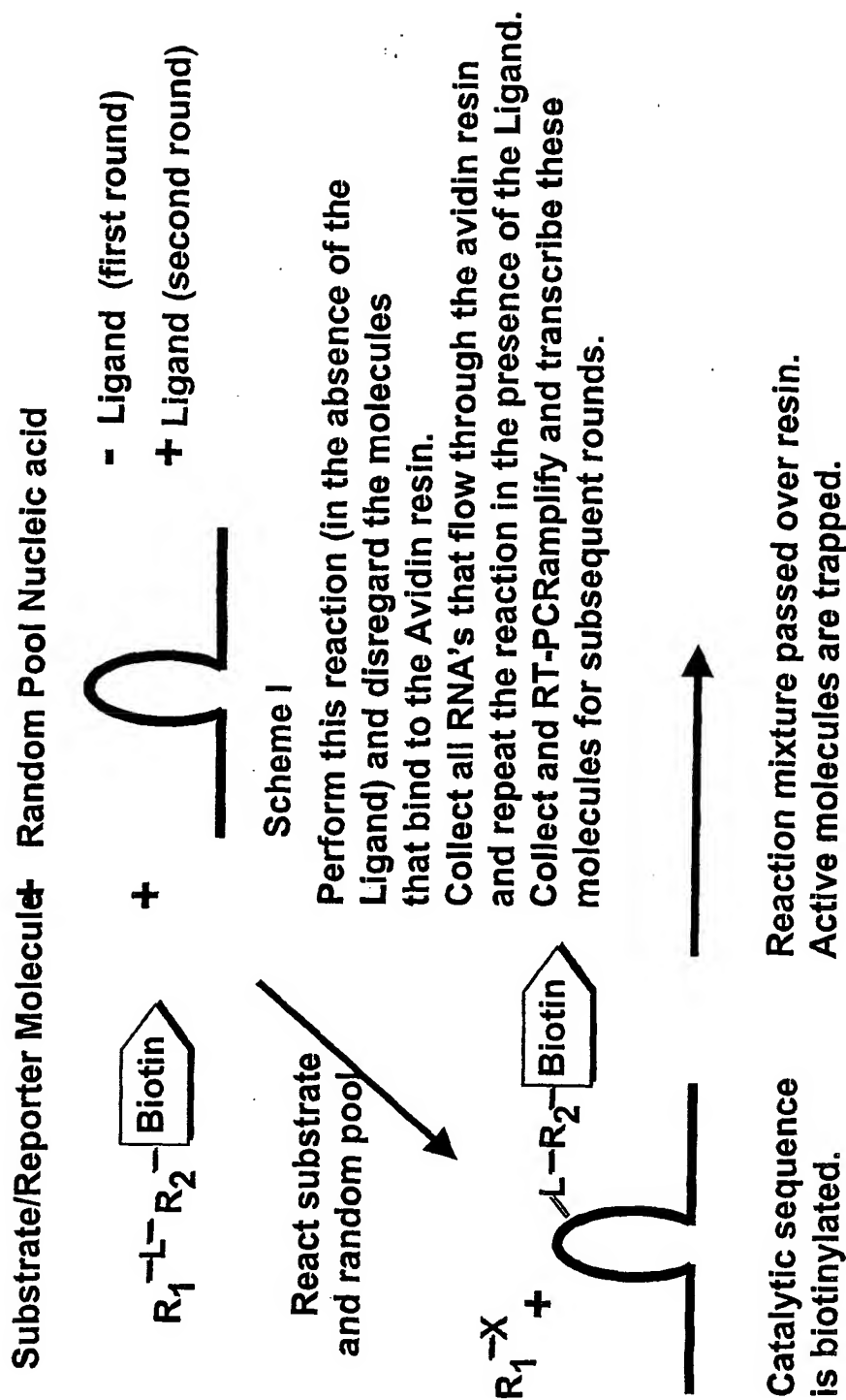
**Figure 16: Ribozyme Diagnostic Screen**  
INHIBITORY FOLDING WITH TARGET RESCUE



**Figure 17a: Auto-ligation Nucleic Acid Sensor Molecules - Selection Scheme**



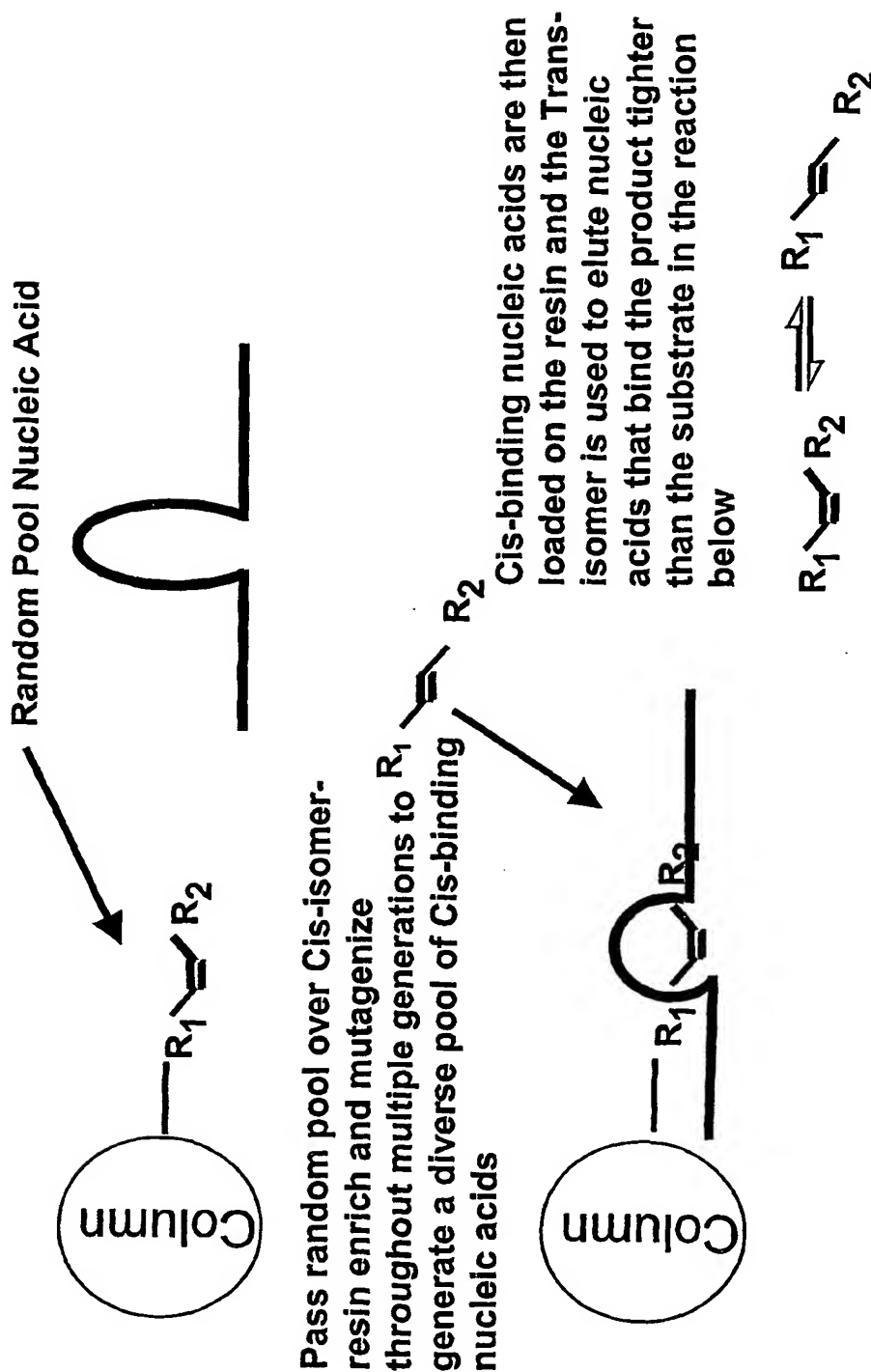
**Figure 17b: Auto-ligation Nucleic Acid Sensor Molecules -  
Ligand Dependent**



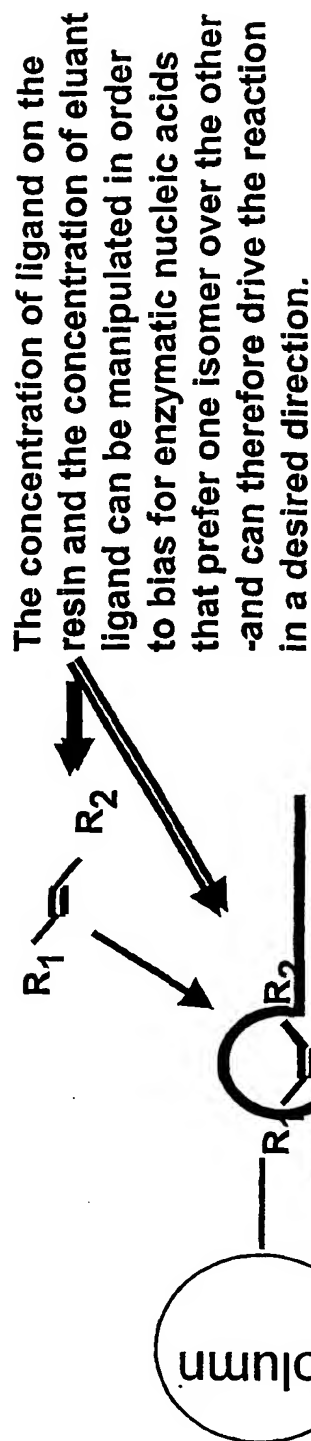




**Figure 18a: Isomerase Nucleic Acid Sensor Molecule - Selection Scheme**



**Figure 18b: Isomerase Nucleic Acid Sensor Molecule -  
Selection Scheme**



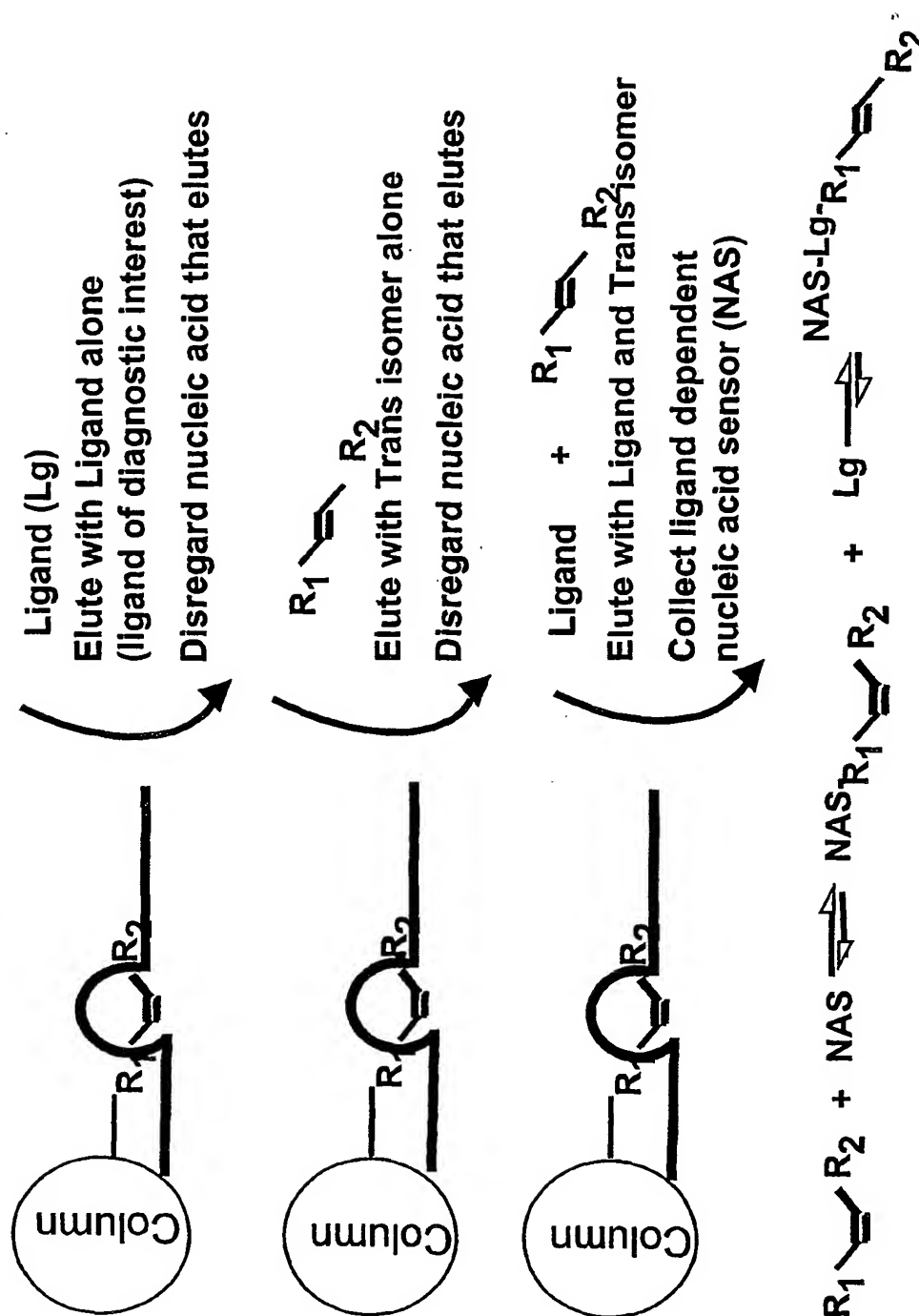
E.g. Selection for CIs-isomer at 100  $\mu\text{M}$  - yield  $\text{cis } K_d = 100$   
 $\mu\text{M}$

Elute with Trans-isomer at 0.1  $\mu\text{M}$  - yield  $\text{trans } K_d = 0.1$   
 $\mu\text{M}$

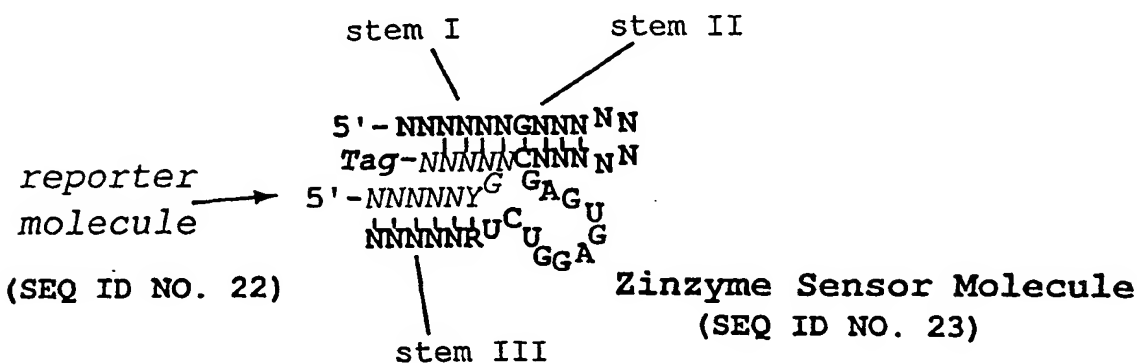
Isolate catalysts for the reaction below



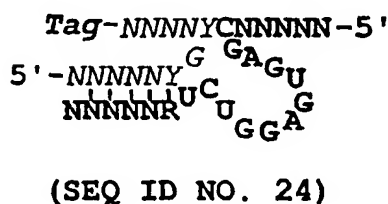
**Figure 18c: Isomerase Nucleic Acid Sensor Molecule -  
Ligand dependent**



## Zinzyme Sensor Molecule for detection of Nucleic Acid



Inactive Zinzyme sensor/  
reporter molecule complex

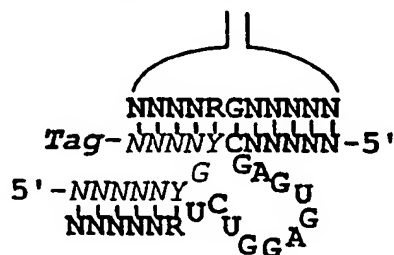


Target Signaling  
Molecule

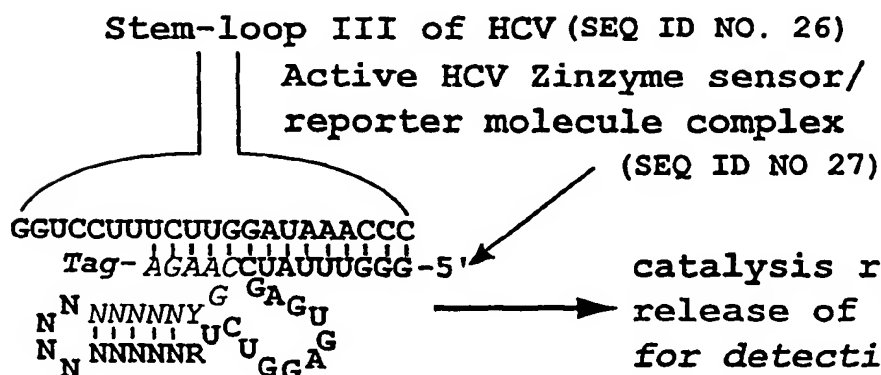


Target Signaling  
Molecule

(SEQ ID NO. 25)



Active Zinzyme sensor/  
reporter molecule complex

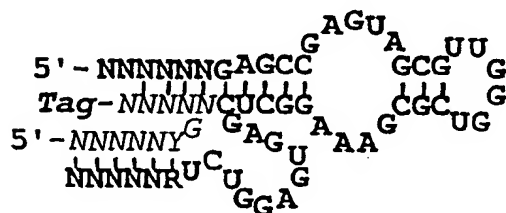
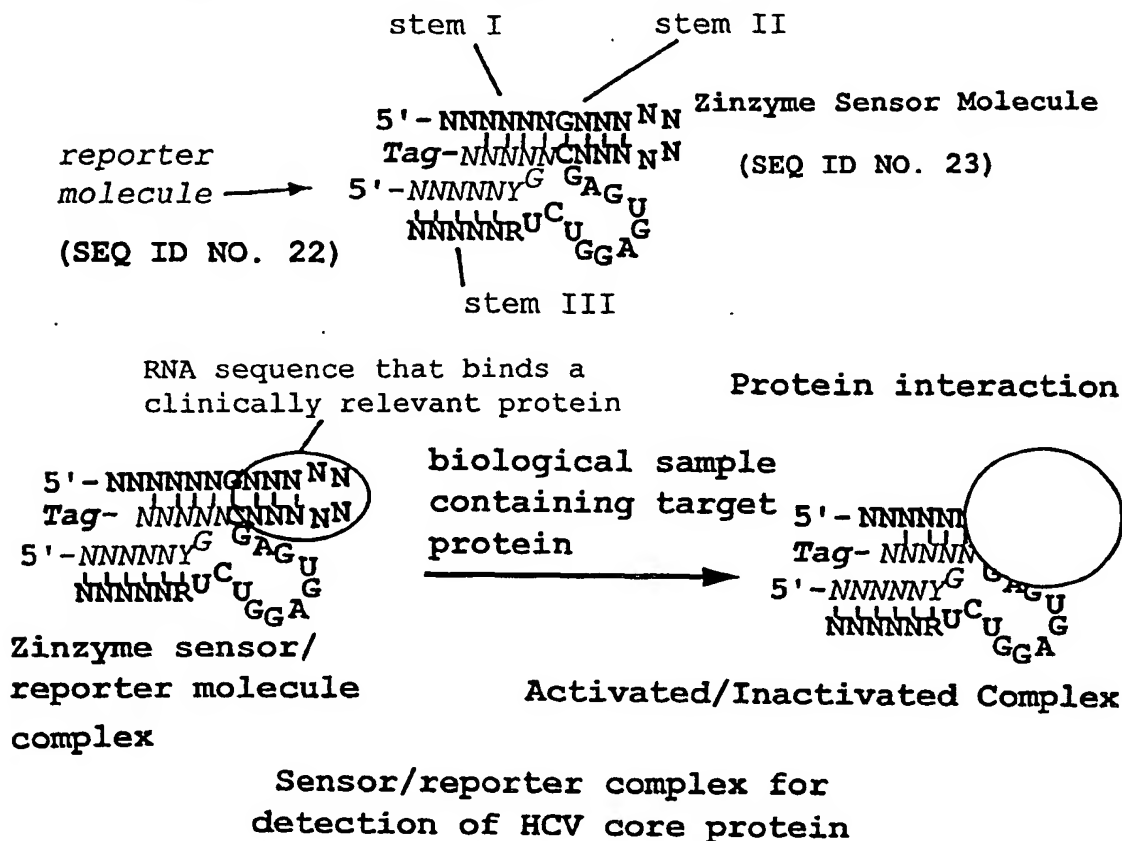


catalysis results in  
release of Tag-AGAAC  
for detection

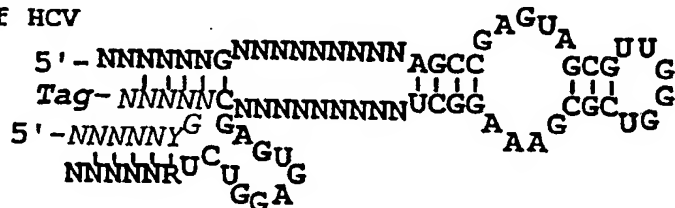
Zinzyme sensor can be attached to solid support/surface,  
for example at the 5'-end

FIG. 19

### Zinzyme Sensor Molecule for detection of Protein



HCV Zinzyme sensor with  
loop IIID of HCV  
(directs the binding of HCV  
core protein)  
(SEQ ID NO 28)



HCV Zinzyme sensor with loop IIID  
of HCV connected via randomized  
linker  
(SEQ ID NO 29)

FIG. 20



# Amplification of signal via use of protein enzyme conjugate

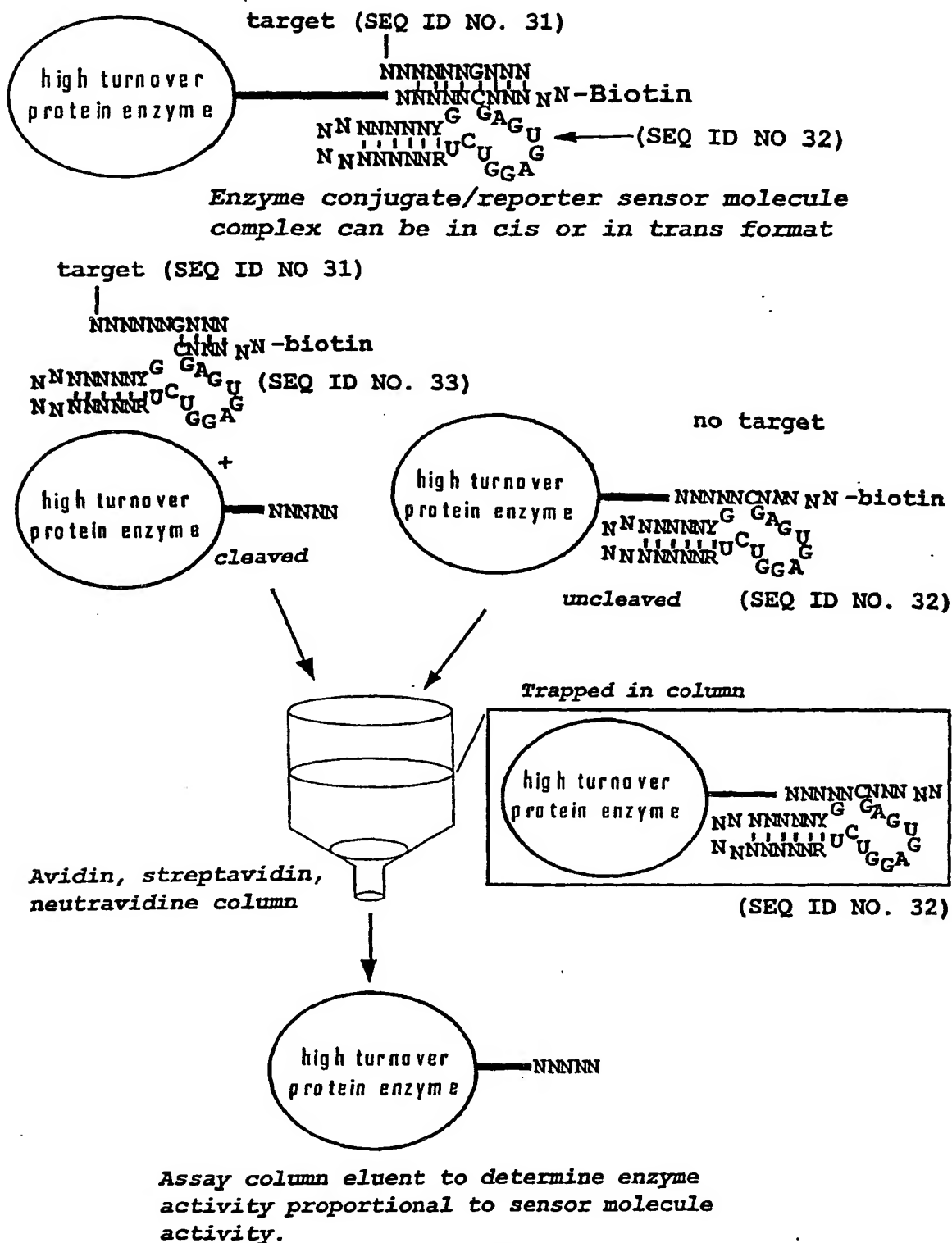
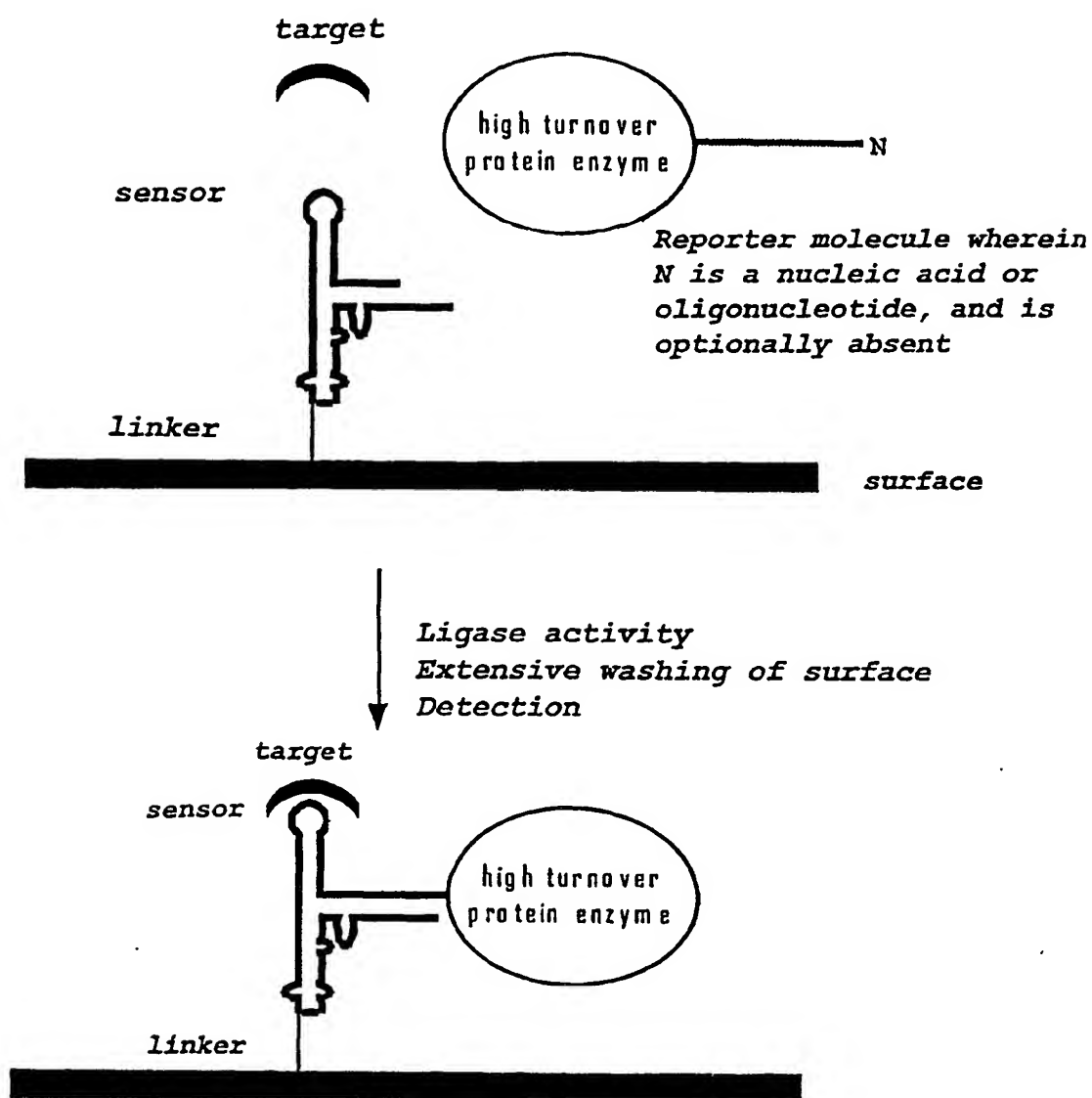


FIG. 22



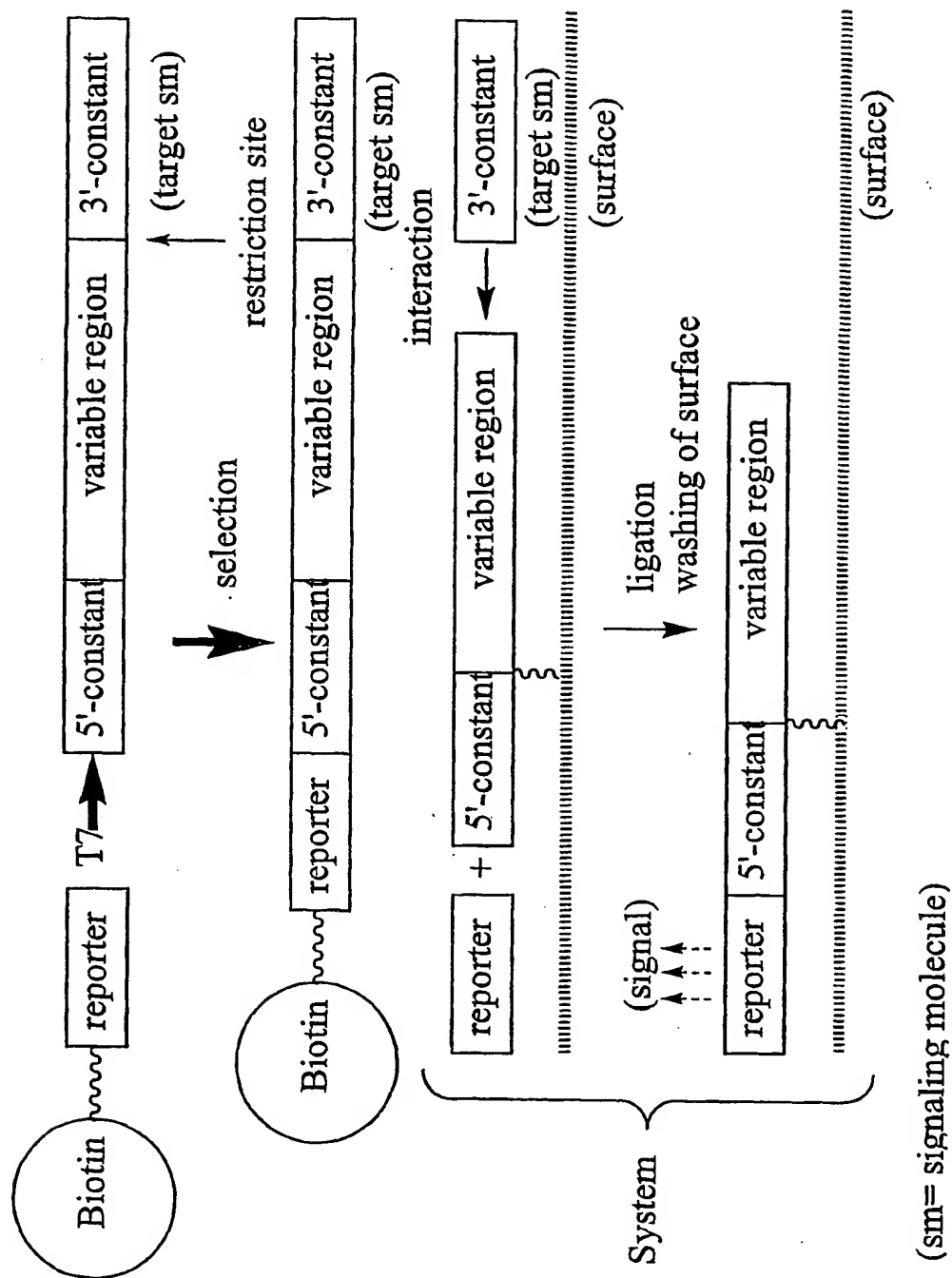
*Ligase Sensor Molecule with enzymatic reporter*



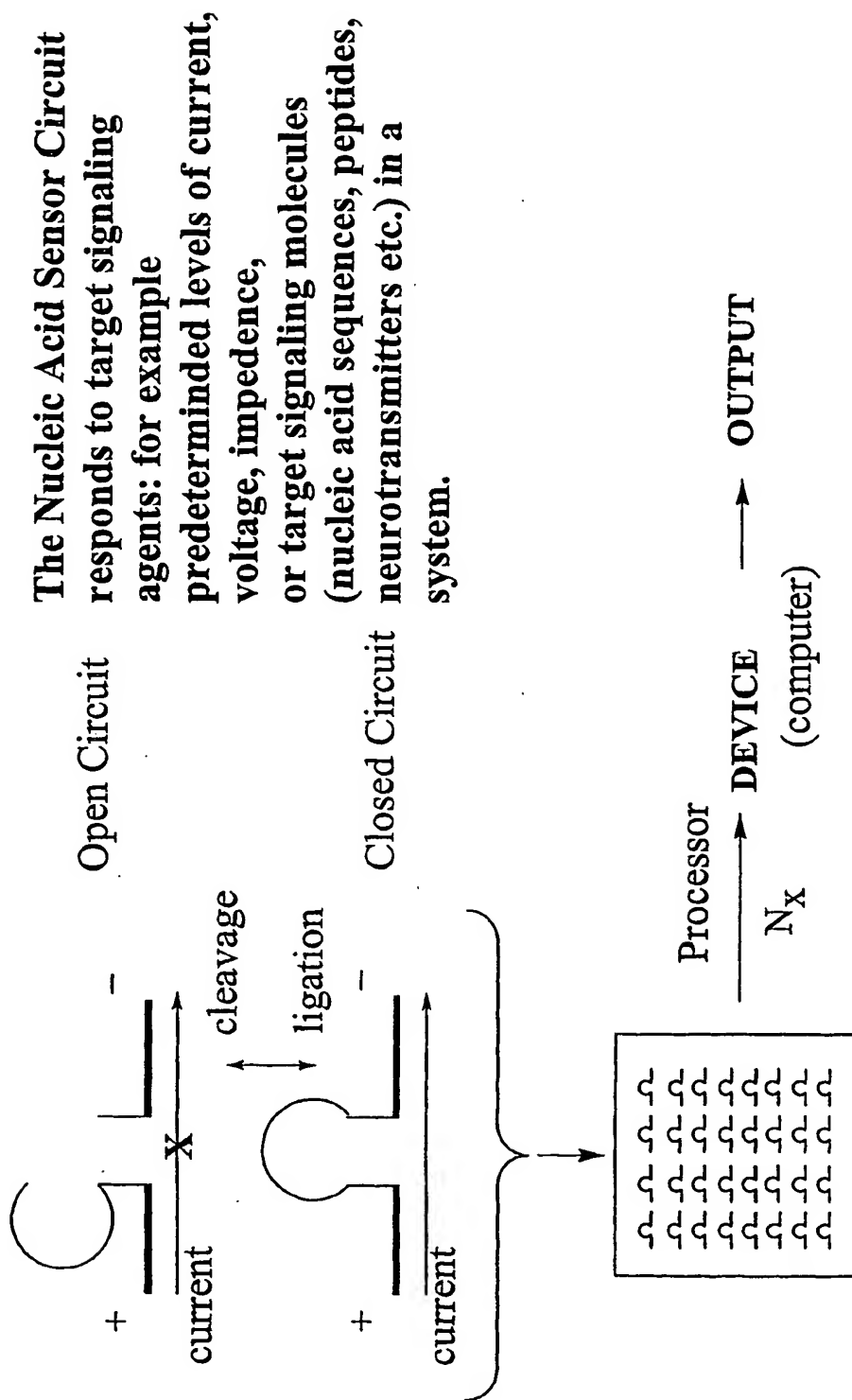
*Alternatively, a fluorescent or chemiluminescent based reporter molecule is used.*

FIG. 23

*Figure 24: Selection of Nucleic Acid Sensor Molecules with Ligase Activity*

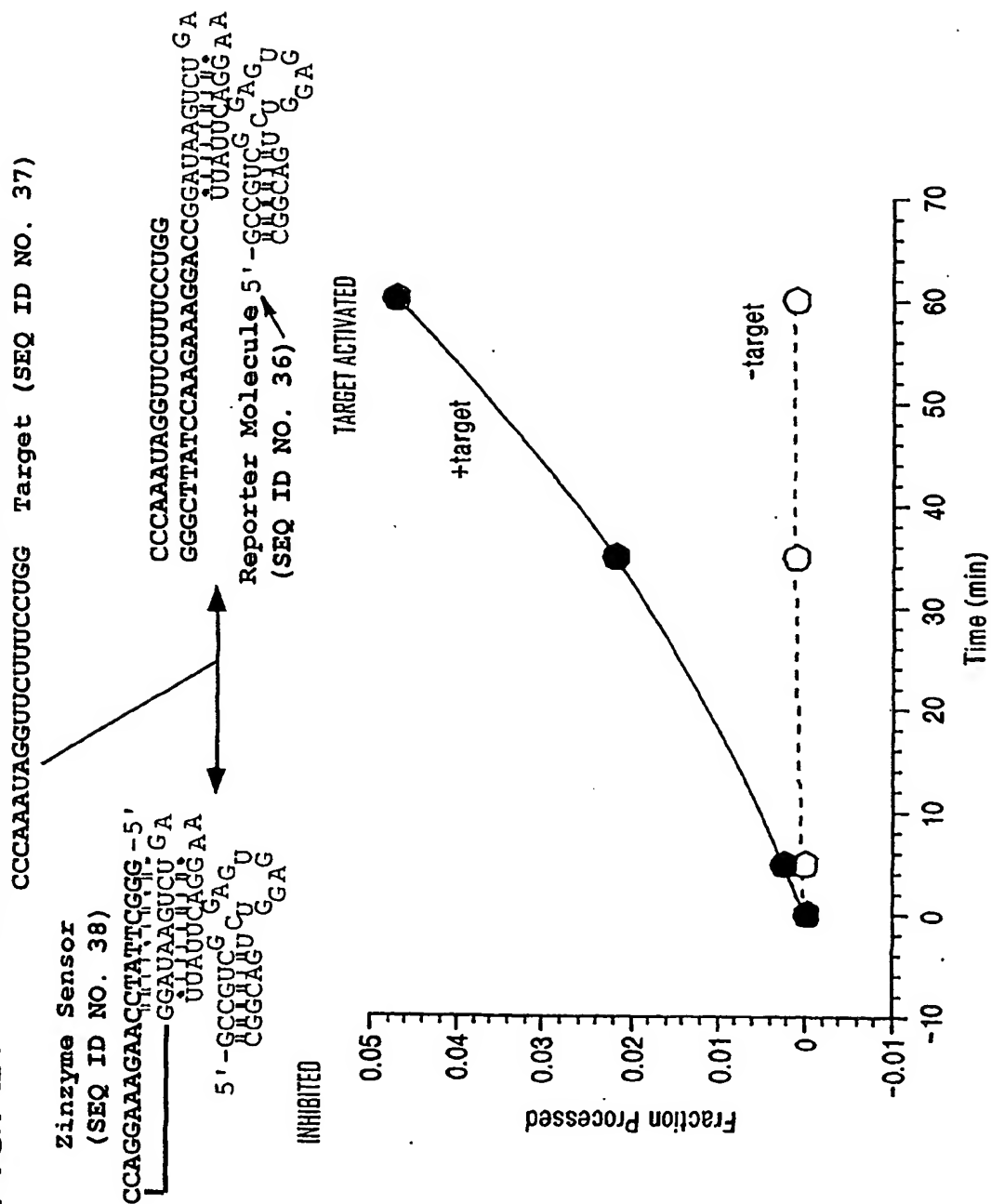


**Figure 25: Nucleic Acid Sensor Molecule-Based Electric Circuit**





**FIG. 27**  
*Target Activation of Zinzyme Sensor Molecule*



*Erk modulated Nucleic Acid Sensor Molecule*

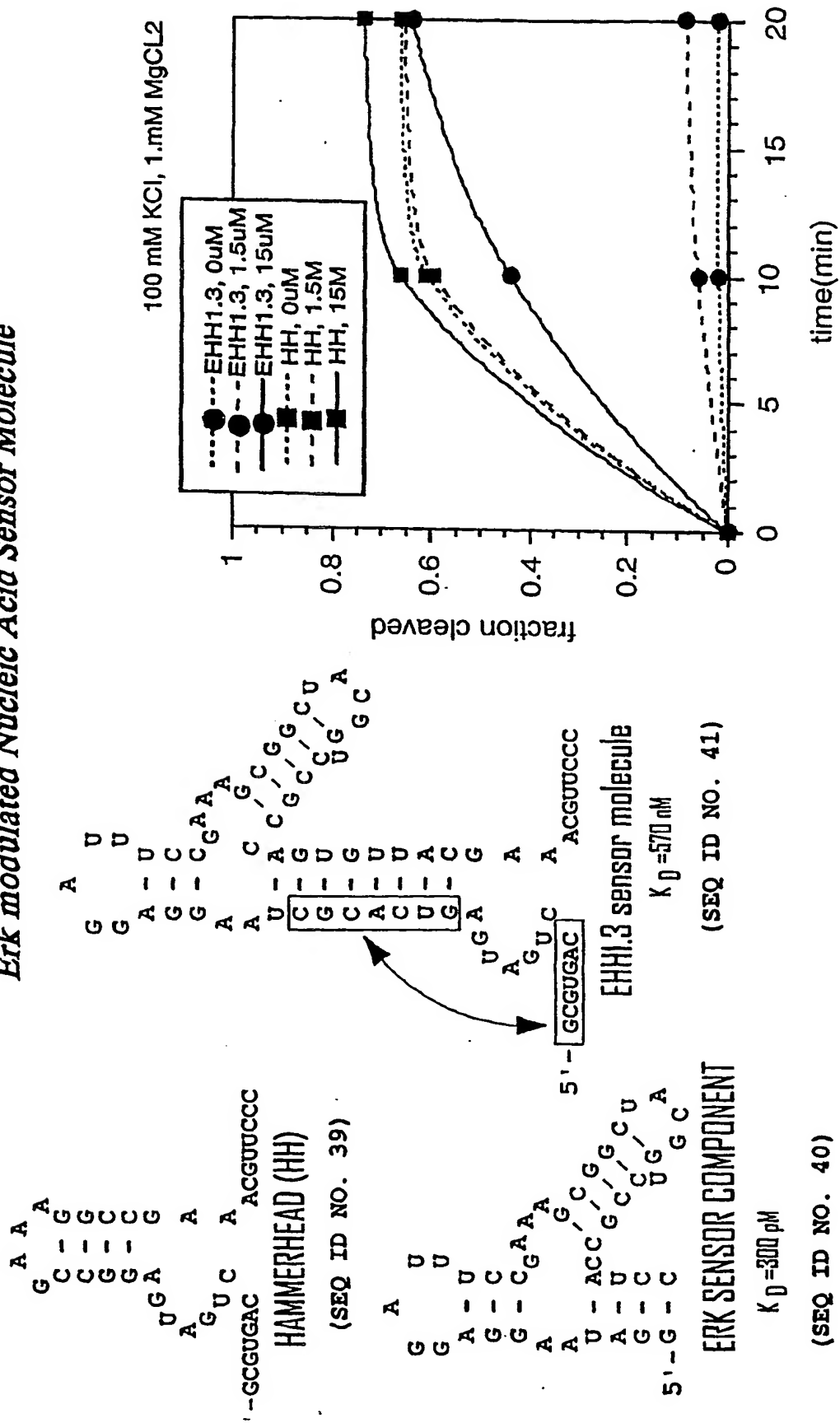
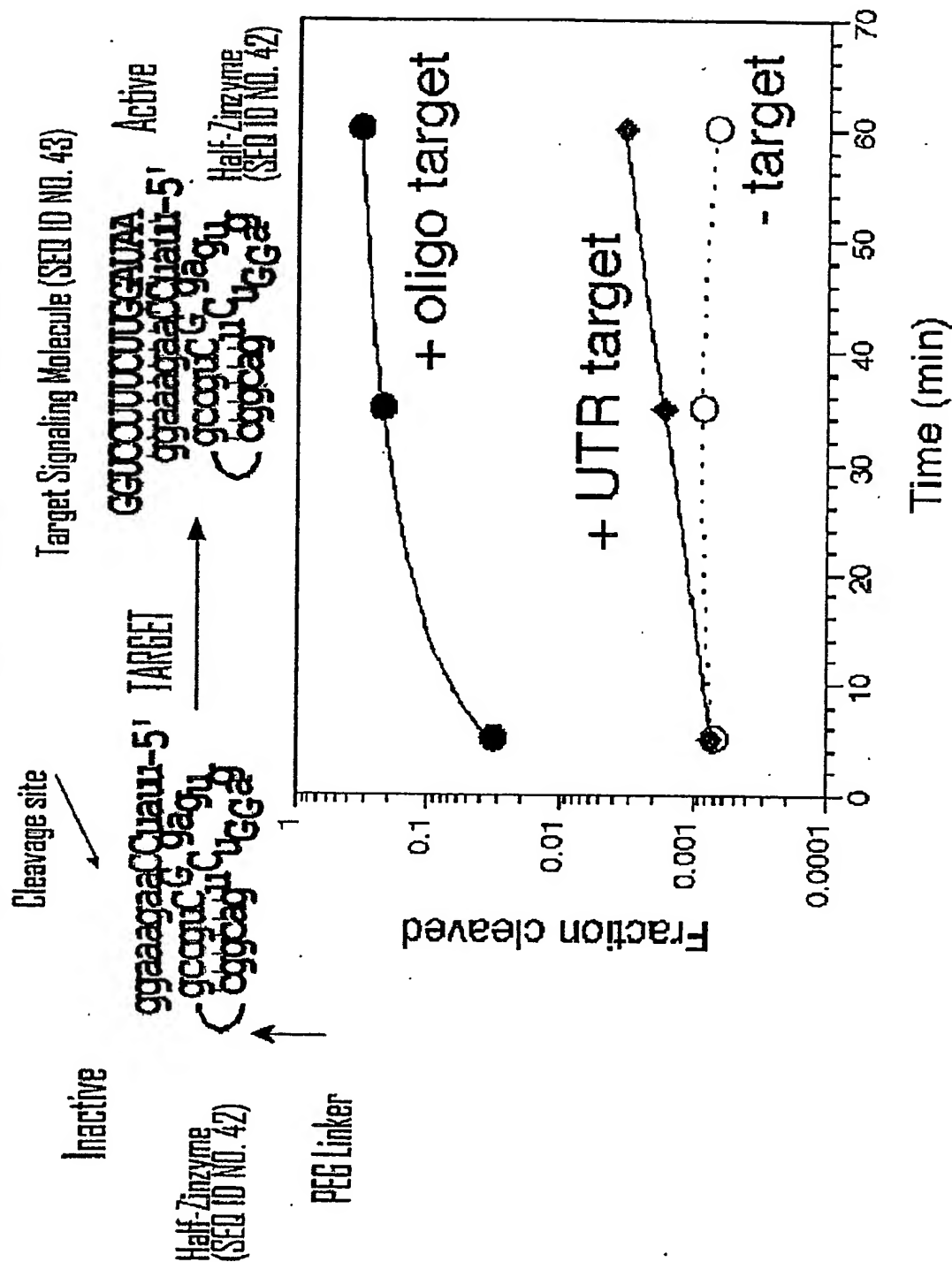


FIG. 28

Figure 29: Half-Zinzyme Construct



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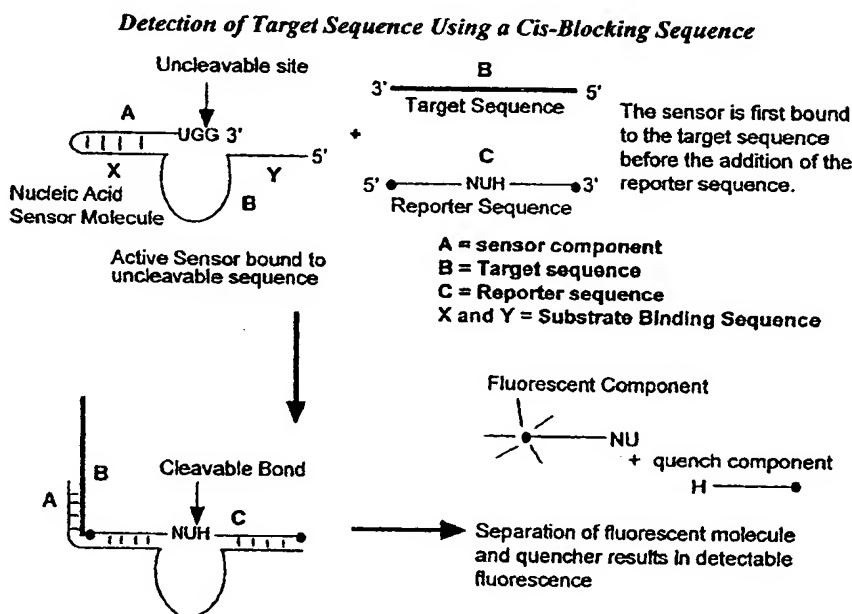
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[Continued on next page]

(54) Title: NUCLEIC ACID SENSOR MOLECULES



(57) Abstract: Nucleic acid sensor molecules and methods are disclosed for the detection and amplification of signaling agents using enzymatic nucleic acid constructs, including hammerhead enzymatic nucleic acid molecules, inozymes, G-cleaver enzymatic nucleic acid molecules, zinzymes, amberzymes and DNAzymes; kits for detection and amplification; use in diagnostics, nucleic acid circuits, nucleic acid computers, and other uses are disclosed.

WO 01/066721 A3





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Minimum documentation searched (classification system followed by classification symbols)

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 20753 A (CECH THOMAS R ;ZHANG BILIANG (US); RIBOZYME PHARM INC (US)) 29 April 1999 (1999-04-29)	1,2,13, 16,22, 23,25, 26,29, 31,36
Y	page 32, line 3 - line 20 claims figures 2,9B	1,2, 16-19, 24, 31-35, 37,58, 60, 62-68, 70-73, 78-80
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☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search

19 December 2001

Date of mailing of the international search report

21 03. 2002

Name and mailing address of the ISA

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ANDRES S.M.

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 01/07163

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 94 13833 A (INNOVIR LAB INC) 23 June 1994 (1994-06-23)  the whole document ---	1,2, 16-19, 24, 31-35,37
Y	WO 98 51819 A (NANOGEN INC) 19 November 1998 (1998-11-19)  page 6, line 28 -page 10, line 22 page 20, line 20 -page 22 claims ---	58,60, 62-68, 70-73, 78-80
A	SOUKUP G A ET AL: "NUCLEIC ACID MOLECULAR SWITCHES" TRENDS IN BIOTECHNOLOGY, vol. 17, December 1999 (1999-12), pages 469-476, XP002926497 ISSN: 0167-7799 ---	
A	WO 98 27104 A (UNIV YALE ;BREAKER RONALD R (US)) 25 June 1998 (1998-06-25) cited in the application page 13, line 10 -page 17, line 14 page 30, line 25 -page 31, line 14 example 3 claims; figure 1 ---	
A	WO 93 09128 A (NANOTRONICS INC) 13 May 1993 (1993-05-13) ---	
A	WO 96 02651 A (RIBOZYME PHARM INC) 1 February 1996 (1996-02-01) ---	
A	WO 96 40723 A (GEN HOSPITAL CORP) 19 December 1996 (1996-12-19) -----	

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US 01/07163

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Although claim(s) 2,16,19,22-24,29-37,44,78,80 (as far as in vivo methods are concerned) are directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

See FURTHER INFORMATION sheet, invention 1.

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 8-10,13-14,17-18,25-26,29-37,48-50,52-57,62-67,  
70-73,78-80 (all partially) and claims 1-2,16,19,  
22-24,39,44,58,60,68,74-75

An enzymatic nucleic acid catalysing a reaction involving covalent attachment or ligation of a reporter molecule to a nucleic acid sensor molecule; A method for detecting a target signaling agent using this system; A kit, a nucleic acid circuit or computer comprising it, and a method for its isolation.

2. Claims: 8-10,13-14,17-18,29-35,48-50,52-57,  
78-80 (all partially) and claims 3,4,11-12,27-28,  
40,45

As for subject 1, where the reaction involves isomerization of a reporter molecule.

3. Claims: 8-10,13-14,17-18,29-35,48-50,52-57,  
78-80 (all partially) and claims 5-7,41-42,46-47,  
76-77

As for subject 1, where the reaction involves phosphorylation or dephosphorylation of a non-nucleotide part of the reporter molecule.

4. Claims: 25,26,29-37,48-50,52-57,62-67,70-73,  
78-80 (all partially) and claims 15,20-21,38,43,51,  
59,61,69

A method involving a nucleic acid sensor molecule comprising an enzymatic nucleic acid capable of cleaving a reporter molecule in the presence of a target signaling molecule; A kit, nucleic acid circuit or computer comprising the sensor molecule.

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 01/07163

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9920753	A	29-04-1999	AU 1187099 A WO 9920753 A2	10-05-1999 29-04-1999
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